A biochemical study of aminopeptidases in the palatal mucosa of the rat following tooth extractions

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Huusko, P. J. A biochemical study of aminopeptidases in the palatal mucosa of the rat following tooth extractions. *Acta Odont. Scand.* 33, 219—225, 1975.

Aminopeptidase activities were studied biochemically from the supernatants of the homogenates prepared from the normal and post extraction palatal mucosa of the rat. The determinations were carried out at 0.5, 1, 2, 4, 8 and 16 hours and 1, 2, 3, 5 and 7 days following extraction using *N*-L-aminoacyl-2-naphthylamines of arginine, glycine, leucine, proline and valine as the substrates. In addition, in the second part of the study the hydrolysis of *N*-L-aminoacyl-2-naphthylamines of alanine, histidine, isoleucine, lysine, phenylalanine, serine, threonine, tyrosine and valine was tested at 1, 3 and 5 days following extraction. The effect of chloride ions (0.2 M NaCl) on the hydrolysis of arginine and lysine derivatives was also tested. The specific activities of aminopeptidases obtained from the extraction-wound side were compared to those of the control side at each time interval. Tooth extraction caused a considerable reduction in the specific activities of aminopeptidases in the surrounding palatal mucosa immediately after extraction (at 0.5 hr.). The specific activities in the wound tissue remained generally below the control levels for 24 hours and reached their maximum within 5 days following extractions. The highest relative increase was obtained with *N*-L-valyl-2-naphthylamine. Arginine aminopeptidase was activated by chloride ions on an average 20 per cent more in the wound tissue than the control. This effect can be considered suggestive of the activation of aminopeptidase B in the post extraction palatal mucosa.

Key-words: Aminopeptidases; wound healing; tooth extraction

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In a previous study aminopeptidase activities were studied histochemically in the palatal mucosa and gingiva of the rat following molar extractions (*Huusko*, 1974). In that study a slight decrease in activity was observed during the first post extraction day, with the most pronounced histochemical staining seen in 4—7 day-old wounds. The results were parallel with both of the substrates used in the incubation medium. i.e. with *N*-L-leucyl- and *N*-L-arginyl-2-naphthylamines. One purpose of the present study was to discover whether the same trend in aminopeptidase activities observed during the extraction-wound healing period could also be shown biochemically in tissue homogenates prepared from the palatal mucosa of the rats. On the other hand, with biochemical methods it is possible to use several different *N*-L-aminoacyl-2-naphthylamines as the substrate. Thus,

Received for publication, March 6, 1975.

a search was made for substrates which are cleaved by aminopeptidases which increase or are activated expressly during the extraction-wound healing period.

MATERIAL AND METHODS

The study was carried out in two parts. In the first series activities were determined at 0.5, 1, 2, 4, 8 and 16 hours and 1, 2, 3, 5 and 7 days after the extractions using three rats at each time interval. The initial age of the animals was 50 days, with an average weight of 195 g. The substrates used in this part were *N*-L-aminoacyl-2-naphthylamines (-2-NA) of arginine (Arg), glycine (Gly), leucine (Leu), proline (Pro) and valine (Val). In the second series the determinations were carried out at time intervals of 1, 3 and 5 days following extraction using four rats in each group. These animals were 55 days old and weighed an average of 205 g. The substrates used in the second part were *N*-L-aminoacyl-2-naphthylamines of alanine (Ala), histidine (His), isoleucine (Ileu), lysine (Lys), phenylalanine (Phe), serine (Ser), threonine (Thre), tyrosine (Tyr) and valine (Val). *N*-L-valyl-2-naphthylamine was included in the second series for the sake of comparison in concequence of the results from the first series. The substrates were obtained from Mann Research Laboratories Inc., New York.

The handling of the rats and the extraction technique are described elsewhere (*Huusko*, 1974). All the left maxillary molars were extracted whilst the rats were lightly anaesthetized with ether. After killing the animals the whole of the palatal mucosa between molar teeth was divided by midline incision and the two halves removed. The right hand piece, coming from the uninjured half of the mouth, served as an interal control for each rat. In the first series the specimens from three rats (control tissues and wound tissues separated) were collected together into 2.5 ml of 0.05 M ice cold phosphate buffer, pH 7.0. In part II the specimens from four rats were collected into 3 ml of the same type of buffer solution. It was necessary to pool samples from 3 and 4 rats in order to obtain sufficient material for all the enzyme assays. The tissue pieces were homogenized with a rotating glass homogenizer (Potter-Elvehjem). The homogenates were centrifuged at 10.600 g for 15 min. at 0—4°C.

The principle of *Goldberg & Rutenburg* (1958) was used for the determinations of aminopeptidase activity from the supernatant fluids. The reaction mixture contained 0.3 ml of 0.05 M phosphate buffer pH 7.0, 0.1 ml of substrate solution (10^{-3} M), 0.1 ml of enzyme solution and 0.1 ml of water (or NaCl solution in special cases). The incubation was carried out at 30°C for 60—90 min., the time depending on the substrate used. The reaction was stopped by adding 0.5 ml of 0.05 % Fast Garnet GBC solution in 1.0 M sodium acetate-acetic acid buffer, pH 4.2, containing 10 % Tween 20. The absorbance of the final reaction mixture was read spectrofotometrically at 525 nm against a mixture, to which water had been added instead of the enzyme. The amount of liberated 2-naphthylamine was calculated from the standard curve prepared in an identical way from free 2-naphthylamine (Sigma Chemical Company, USA). In order to elucidate possible appearance of aminopeptidase B, which specifically cleave arginine and lysine derivatives and which is strongly activated by chloride ions (*Hopsu, Mäkinen & Glenner*, 1966; *Hopsu-Havu & Jansen*, 1968), the hydrolysis of *N*-L-arginyl- and *N*-L-lysyl-2-

Table I. *The specific activities of the enzymes acting on N-L-aminoacyl-2-naphthylamines used in the first series of the study. The determinations are made from the supernatants of the homogenate prepared from the specimens of three rats at each time interval. The results are multiplied by 10^3. W = wound-side activity, C = control side activity*

substrates		Time after extraction hours 0.5	1	2	4	8	16	days 1	2	3	5	7
N-L-Arg-2-NA	W	2.77	4.71	6.34	5.92	6.97	9.08	9.58	11.41	13.13	13.87	14.61
	C	7.49	7.35	7.71	8.51	9.83	10.29	9.69	9.31	9.82	9.88	10.96
N-L-Arg-2-NA	W	6.08	7.31	9.46	9.92	11.50	15.68	16.30	18.85	20.16	22.11	24.03
with NaCl	C	11.32	10.46	11.48	10.97	13.93	14.04	13.81	14.66	14.05	14.84	16.58
N-L-Gly-2-NA	W	1.18	1.66	2.11	2.17	1.78	2.50	2.50	3.18	3.23	3.32	3.41
	C	2.79	2.57	2.69	3.08	2.87	3.33	2.93	3.12	2.78	2.72	3.76
N-L-Leu-2-NA	W	2.25	3.57	4.97	5.29	5.75	7.75	8.27	10.03	8.61	10.58	11.59
	C	5.85	5.49	7.18	6.31	8.38	8.90	8.50	8.19	8.00	7.75	8.85
N-L-Pro-2-NA	W	0.74	1.50	2.01	1.86	1.68	2.23	2.48	3.09	2.92	2.82	3.20
	C	2.42	2.27	2.61	2.80	2.74	2.94	2.85	2.71	2.17	2.37	2.65
N-L-Val-2-NA	W	2.57	2.48	2.94	4.07	3.54	6.22	5.85	6.46	5.24	5.91	6.72
	C	2.90	2.33	3.51	3.34	3.57	4.53	4.18	3.95	3.39	3.42	3.42

naphthylamines was also tested in the presence of NaCl (0.2 M). The protein concentrations of the supernatant fluids were measured using the Folin-Giocalteau method as presented by *Layne* (1957). The standard curve was prepared from bovine serum albumin obtained from Sigma. Double determinations were always performed to verify the results, both in the activity and protein determinations.

RESULTS

First series. The specific activities of the enzymes ($\times 10^3$) hydrolyzing *N*-L-aminoacyl-2-naphthylamines used as the substrates in the first part are expressed in Table I. The protein concentrations of the supernatant fluids are expressed in Table II. The most rapidly hydrolyzed substrate was *N*-L-arginyl-2-naphthylamine, which was followed by the others in the order *N*-L-leucyl-, *N*-L-valyl-, *N*-L-glycyl- and *N*-L-prolyl-2-naphthylamines. The order was the same both on the control and on the wound sides. In Fig. 1 the enzyme activities from the wound side are compared with the activities on the control side at each time interval. The activities on the wound side had markedly decreased at 0.5 hr., but a pronounced intensification occurred during the first two hours. In most cases the activities on the wound side remained below the control level

Table II. *The protein concentrations (mg/ml) of the supernatants used for the activity determinations in the first series of the study*

Time interval	Wound side	Control side
0.5 hour(s)	0.444	0.356
1	0.424	0.376
2	0.389	0.376
4	0.450	0.372
8	0.423	0.354
16	0.475	0.382
1 day(s)	0.459	0.380
2	0.435	0.374
3	0.472	0.402
5	0.526	0.376
7	0.529	0.386

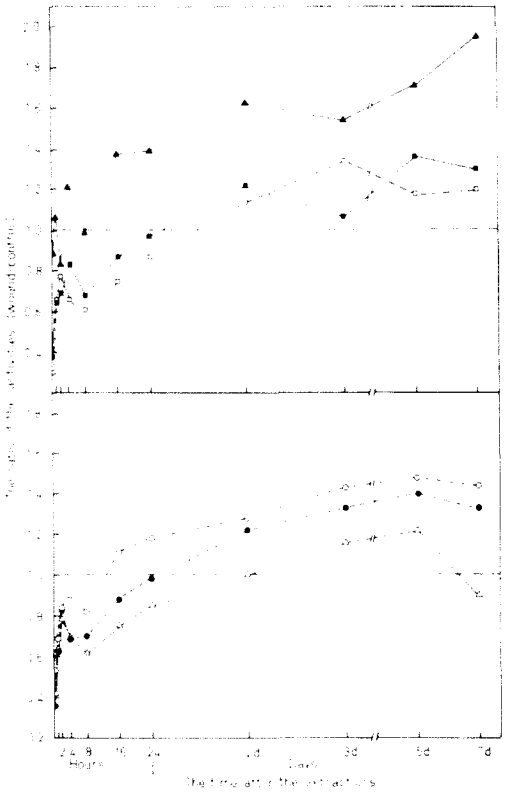

Fig. 1. The development of the ratios of wound activity: control activity during the healing period of the tooth extraction wound studied in the first part of the study. The curves indicate the development at each substrate used.

during the first post extraction day. The highest relative increase occurred in 3—5 day-old wounds. *N*-L-valyl-2-naphthylamine produced results differing from the others, in that the initial decrease was not so marked as with the other substrates and the control level was exceeded first at 1 hr. and again at 4 hrs. In 16-hour wounds the activity of the wound side was markedly higher than that of the control side. At the seventh post extraction day the hydrolysis of *N*-L-valyl-2-naphthylamine was nearly twice that of the control. With the other substrates at this time interval the ratio ranged from 0.90 to 1.44.

The effect of sodium chloride on arginine aminopeptidase both on the wound and on the control sides at each time interval is presented in Table III. The effect was always activation and was an average of 20 per cent higher on the wound side when the results at 0.5 hr. are disregarded (at this time the activation was exceptionally high on the wound side).

Before commencing the first part of this study 2 preliminary experiments were carried out using slightly different techniques from those described in Material and methods and in addition, the only time intervals examined were 1, 4 and 16 hours and 1, 3 and 7 days. Despite these differences in techniques the results from these experiments were quite similar to those of the larger series.

Second series. In order to determine whether the result obtained at 24 hrs. using *N*-L-valyl-2-naphthylamine as substrate were really exceptional, and whether it would be possible to obtain more dramatic results in older wounds with

Table III. *The per cent increase caused by sodium chloride in the hydrolysis of N-L-arginyl-2-naphthylamine at the different intervals both on the wound- and control sides*

	Time intervals										
	hours						days				
	0.5	1	2	4	8	16	1	2	3	5	7
wound side	119.4	55.2	49.2	67.5	64.9	72.6	70.1	65.2	53.5	59.4	64.4
control side	51.1	42.3	48.8	28.9	41.7	36.4	42.5	57.4	43.0	50.2	51.2

Table IV. *The specific activities of the enzymes acting on the substrates used in the second series of the study. The determinations are made from the supernatant of the homogenate prepared from the specimens of four rats at each time interval. The results are multiplied by 10^3. W = wound-side activity, C = control side activity. The protein contents are also included in this table*

Substrates		Time intervals 1 d	3 d	5 d
N-L-Ala-2-NA	W	11.79	17.15	17.47
	C	13.08	11.25	11.75
N-L-His-2-NA	W	1.70	2.56	2.22
	C	1.88	1.42	1.59
N-L-Ileu-2-NA	W	1.21	1.33	1.27
	C	1.11	0.94	0.99
N-L-Lys-2-NA	W	7.73	11.65	11.44
	C	8.96	8.08	7.93
N-L-Lys-2-NA	W	8.44	12.35	12.50
with NaCl	C	9.54	8.18	8.33
N-L-Phen-2-NA	W	11.01	16.66	15.44
	C	13.08	9.92	9.68
N-L-Ser-2-NA	W	1.16	1.65	1.73
	C	1.48	1.37	1.41
N-L-Thre-NA	W	1.67	2.45	2.50
	C	2.08	1.81	1.81
N-L-Tyr-2-NA	W	5.08	5.53	5.60
	C	6.20	6.19	6.15
N-L-Val-2-NA	W	5.28	6.06	5.81
	C	4.06	2.97	2.94
Protein mg/ml	W	0.448	0.476	0.472
	C	0.372	0.336	0.336

some other substrates, eight new substrates were tested in 1, 3 and 5 day-old wounds. These results are expressed in Table IV. In Fig. 2 the columns illustrate the ratios of the activities (wound: control). Only *N*-L-isoleucyl- and *N*-L-valyl-2-naphthylamine cleaving enzymes exceeded the control level at 1 day, the others remaining more or less below that. Generally, each substrate cleaving enzyme was about on the same level at 3 and 5 days; one exception was *N*-L-histidyl-2-naphthylamine cleaving enzyme, which seemed to have been rather more active in the 3-day wounds. The relative increase of *N*-L-valyl-2-naphthylamine cleaving enzyme was by far the highest also in this part of the study. In the hydrolysis of *N*-L-lysyl-2-naphthylamine chloride ions caused an average increase of 8.2 per cent on the wound side and 3.6 per cent on the control side.

DISCUSSION

On the basis of the results the following general conclusions can be drawn:

— Tooth extraction considerably reduces aminopeptidase activity in the surrounding mucosa for the first hours following extractions.

— The wound-side activities reach the control level at about the second post extraction day and seem to reach their maximum within five days following extraction.

— The enzyme (or enzymes) acting on *N*-L-valyl-2-naphthylamine had the relatively most pronounced increase on the extraction side. On the other hand, the initial decrease in the activity

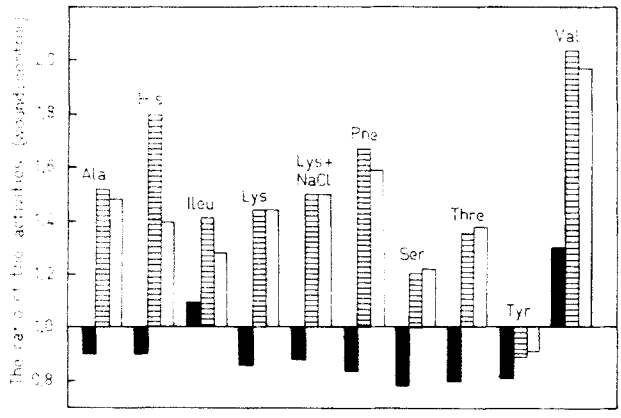

Fig. 2. The ratios of wound activity: control activity obtained with the substrates used in the second part of the study at

after the extractions.

of this (these) enxyme(s) was the slightest and this activity returned most rapidly to the control level.

On the basis of the chloride-ion effect on arginine aminopeptidase it is logical to expect that aminopeptidase B partakes in the normal metabolic function of the palatal mucosa and gingiva of the rat. In addition, because the activating effect was 20 per cent higher on the wound side as compared with the control, it is also expected that the increase or activation of aminopeptidase B occurs during extraction-wound healing. It has been established in a biochemical study including molecular permeation chromatography and characterization of the enzyme acting on *N*-L-arginyl-2-naphthylamine that the normal gingiva of the rat contains an enzyme identical or very closely related to aminopeptidase B, which increases somewhat during the healing after mechanical occlusal stress (*Mäkinen & Virtanen*, 1974).

The previous histochemical observations by the author are confirmed by the present study. The greatest discrepancy is at 0.5 hr, when histochemically a slight increase seemed to have occurred. Perhaps the total activity of the extraction side of the palatal mucosa was also decreased in that histochemical study, but intensified staining near the gingival pocket bottom was so distinct that it was registered as increased activity.

The high values of specific activities obtained in 3—5 day-old extraction wounds could be connected with fibroblastic proliferation, which has been observed to be in active phase during that period (*Todo*, 1968; *Huusko*, 1974). The intensification of the activities appearing after the initial decrease might be caused by enzyme biosynthesis occuring in wound tissue itself. This assumption has been manifested also in connection with studies on healing skin wounds (*Mäkinen & Raekallio*, 1967, 1968). The invading leucocytes can also participate in increase of aminopeptidase activity in the extraction-wound tissue. The possible role of leucocytes will be elucidated in following papers.

As it can be seen from Tables II and IV, the protein concentrations of wound supernatants are always higher than those of the controls. Initially this is probably due to the accumulation of plasma proteins in the injured mucosal tissue. Later it is probably due to the tissue growth associated with the healing process. Only a

minimal part of proteins in the supernatants fluids are responsible for aminopeptidase activities. Similarly, only a minimal part of the protein increase in wound tissue is enzyme protein. Thus, when the results are calculated as specific activities, the activity values in wound tissue might be considered too low compared with the controls. However, even when the results were expressed as μmol/min/sample the curves were similar to those of specific activity, except that all the changes took place a little earlier and the difference between control and injured tissue were greater. Furthermore, the relationship between the different substrates is not altered.

Acknowledgement. Lauri Nieminen, M.Sc., is acknowledged for practical assistance. For technical assistance I am grateful to Miss Ulla Helinä Sammallahti and Mrs. Toini Tolvanen. This study was supported by grants from Sigrid Juselius Foundation to the department of Forensic Medicine, University of Turku, Finland and by a grant from Finnish Dental Society to the author.

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