ACTA CHEMICA SCANDINAVICA 21 (1967) 761–767

# Studies on the Formation of Aminopeptidase-like Enzymes in Regenerating Wound Tissue

PIRKKO-LIISA MÄKINEN and JYRKI RAEKALLIO

*Department of Forensic Medicine, University of Turku, Turku 3, Finland*

The activity of aminopeptidase-like enzymes from the regenerating wound tissue of the rat has been studied. The enzyme activity hydrolysing 2-naphthylamides of L-leucine and DL-alanine began to increase about 2 h after the injury, whereas that affecting 2-naphthylamide and *p*-nitroanilide of L-lysine was increased as soon as 1 h after the injury. The wound enzymes were fractionated by ammonium sulphate precipitation and by CM-cellulose chromatography. The stability of the enzymes involved has been studied. The obtained results indicate the existence of different, wound specific aminopeptidase-like enzymes.

There are only a very few studies concerning the chemistry of aminopeptidase-like enzymes participating in wound healing. Monis [1] has, however, studied aminopeptidase activities in the wound healing, but these studies have been performed from the third day on. Raekallio [2–5] was the first to show that increased enzyme activity was seen even during the very first hours of wound healing. He has determined various enzyme activities by histochemical methods and found, *e.g.*, that the enzyme activity demonstrable by L-leucyl-2-naphthyl-amide was increased as soon as 2 h after the injury. Raekallio and Mäkinen [6] have confirmed this by biochemical methods. It is further possible that during the early phases of wound healing there exist also such enzyme activities as will disappear later on.

This paper describes studies supplementing the investigations mentioned above. The essential part of this study concerns the wound tissue, because the working hypothesis has been that the enzyme pattern of the wound tissue differs qualitatively and quantitatively from that of the normal tissue.

## MATERIALS AND METHODS

*Reagents.* The 2-naphthylamides of various amino acids used in these studies were purchased from Mann Research Laboratories Inc., New York, USA, except for DL-alanyl-2-naphthylamide, which was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, USA. Fast Garnet GBC Salt (diazotized 4-amino-3,1′-dimethylazobenzene) was a product of G.T. Gurr, London, England, and tris(hydroxymethyl)amino-

methane from Sigma Chemical Co. (St. Louis, Miss., USA). Other reagents used in this study were purchased from E. Merck AG (Darmstadt, Germany) and were of analytical grade or the equivalent.

*Procedures.* 4 to 9 months old albino rats of both sexes were used without anaesthesia. Square skin wounds, 1 cm$^2$, were cut in a shaved dorsal area. The skin inside the square was excised. After decapitation, skin flaps, each containing one wound, were cut to four pieces. The wound edge was carefully adjusted as linear as possible and the specimens were frozen between two plates of solid carbon dioxide. The skin specimens were fastened to a specimen holder plane of a microtome with distilled water, and 100 sections of 16 $\mu$ were cut in a cryostat (International Equipment Company, model CTD) from the wound edge. The sections were homogenized immediately with Ultra-Turrax top drive homogenizer (Janke & Kunkel, KG, Stauffen i. Br., Germany) in 0.1 M tris-HCl buffer, pH 7.15, for 5 sec.

The so-called control wounds were made on the same rats 15 min before the decapitation. These control wounds were made and the tissue was prepared in exactly the same way as the other wounds. It was necessary to use controls because it is obvious that the destruction of the cells at the edge of the wound produces bleeding and local changes in permeability, ionic strength, pH, and in the structure of proteins. It is apparent that the above mentioned changes take place particularly in the so-called central zone (200—500 $\mu$) near the wound edge where enzyme activity is markedly decreased as described by Raekallio.[2-5] Consequently, these phenomena took place in every wound preparation. The results obtained with the control wounds were used in drawing the curves describing changes in enzyme activity as a function of time.

The enzyme preparation used consisted of a crude protein solution obtained after centrifuging (23 500 *g*, 10 min) the tissue homogenate. All of the substrates used were hydrolysed most rapidly by this crude preparation at the pH range 7.0—7.5 when the substrate concentration was $10^{-4}$ M.

*Determination of enzyme activity.* The reaction mixture consisted of 400 $\mu$l 0.1 M tris-HCl buffer, 100 $\mu$l water and 100 $\mu$l $10^{-3}$ M substrate solution. All reactions were carried out at pH 7.15. The incubations were run at 37°C. When the enzymatic reaction was completed, 300 $\mu$l of freshly prepared Tween-diazonium salt mixture was added to each of the tubes. The reagent consisted of 10 % Tween 20 and 0.1 % Fast Garnet GBC Salt in 1 M sodium acetate-acetic acid buffer, pH 4.2. Full development of the color required about 5 min. The optical density was determined by Beckman DB double beam spectrophotometer using the wavelength 525 m$\mu$. Enzyme activities have been generally expressed as extinctions.

*Determination of proteins.* Protein concentrations were measured using the Folin-Ciocalteu method as presented by Layne.[7] Bovine serum albumin was used as a standard.

*Preparation of gel filtration and ion exchange columns.* The gel filtration was conducted on a Sephadex G-200 column. In the preparation of gel and the packing of the column, the instructions of the manufacturer have been followed (Pharmacia, Uppsala, Sweden).

*CM-cellulose chromatography* was carried out on a column packed with a product of Schleicher & Schüll (Dassel/Kr. Einbeck, Germany). The commercial cellulosic adsorbent was sieved into more homogeneous fractions and that between 100 and 140 mesh was separated for further treatment. In preparation of the material, in packing the column, and in conducting the fractionations, the instructions presented by Peterson and Sober [8] have been followed except for the pressure applied in the packing. The column was packed under a pressure accomplished by a 120 × 1 cm glass tube. The suspension was poured into the tube and the level of the mixture was kept as high and constant as possible during the packing.

## RESULTS

Fig. 1 describes the dependence of enzyme activities on the age of the wound. The activity demonstrable by 2-naphthylamides of L-leucine and DL-alanine began to increase after 2 h, whereas that by 2-naphthylamide and *p*-nitroanilide of L-lysine increased as soon as 1 h after the injury. The results show that in the wound tissue the formation of enzymes possessing different

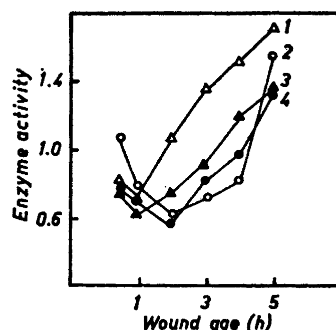


*Fig. 1.* Changes in the activity of aminopeptidase-like enzymes in rat skin wounds during the early phases of healing. The activities are expressed as the ratio of the activity of the wound to that of the control wound. 1 = L-lysyl-*p*-nitroanilide, 2 = L-leucyl-2-naphthylamide, 3 = L-lysyl-2-naphthylamide, 4 = DL-alanyl-2-naphthylamide.

function and structure starts at different phases of wound healing. The term "formation" is used in this connection. Another explanation of the increased enzyme activities would be some kind of activation.

Considerable differences in enzyme activities were obtained with different substrates two days after the injury. This fact is shown in Table 1. According to the observed hydrolysis rates the substrates could be divided into two main groups: a) Substrates which are hydrolysed at least twice as rapidly by the wound tissue enzymes as by those of the control wound. This group included 2-naphthylamides of L-valine, L-threonine, L-isoleucine, L-methionine, L-phenylalanine, L-alanine, L-leucine, L-arginine, L-histidine, and L-cystine.

*Table 1.* The hydrolysis of 2-naphthylamides of certain amino acids by enzymes of the control wound and of a two day-old wound. The activities are expressed as specific activities, the hydrolysis rate of L-methionyl-2-naphthylamide indicated as 100.

| 2-Naphthylamide of | Control wound | Two day-old wound | Two day-old wound / Control wound |
|---|---|---|---|
| L-Methionine | 100 | 438 | 4.4 |
| L-Lysine | 95 | 134 | 1.4 |
| L-Alanine | 64 | 138 | 2.1 |
| L-Leucine | 55 | 104 | 1.9 |
| L-Phenylalanine | 45 | 124 | 2.8 |
| L-Arginine | 37 | 68 | 1.8 |
| L-Ornithine | 18 | 30 | 1.7 |
| L-Tyrosine | 16 | 20 | 1.2 |
| L-Tryptophane | 10 | 15 | 1.5 |
| L-Serine | 9.0 | 11 | 1.3 |
| L-Proline | 8.1 | 11 | 1.4 |
| L-Valine | 7.9 | 196 | 25.0 |
| Glycine | 7.7 | 12 | 1.5 |
| L-Isoleucine | 6.9 | 62 | 9.0 |
| L-Cystine | 6.7 | 13 | 1.9 |
| L-$\beta$-Glutamic acid | 5.1 | 7.8 | 1.5 |
| L-$\gamma$-Glutamic acid | 2.3 | 2.0 | 0.9 |
| L-OH-proline | 2.2 | 1.6 | 0.7 |
| L-$\alpha$-Asparaginic acid | 2.2 | 2.4 | 1.1 |
| L-Threonine | 1.5 | 19.2 | 12.8 |

b) Substrates that are hydrolyzed at an about equal or at a lower rate by the wound tissue enzymes than by those of the control wound.

During the wound healing the greatest increase in enzyme activity was found by using L-valyl-2-naphthylamide. Thus enzyme(s) hydrolysing this substrate, and also those hydrolysing 2-naphthylamides of L-methionine, L-threonine, and L-isoleucine, would be especially characteristic of the wound tissue in question.

Because of the especially great increase in the enzyme action on 2-naphthylamides of some amino acids it was assumed that at least a part of this increased activity was due to the formation of one or more specific "wound enzymes". To elucidate this, wound samples of various ages were fractionated through CM-cellulose columns using a sodium chloride gradient from 0 to 1.5 M. L-Valyl-2-naphthylamide was chosen as the substrate because the greatest increase in enzyme activity in the wound tissue was demonstrable by it. Fig. 2 shows the results obtained from the normal and wound tissue enzyme fractionations. It appears that the activity on L-valyl-2-naphthylamide was separated into three main peaks and that the wound tissue enzymes are able to hydrolyze this substrate more rapidly than those in the normal tissue. The figure further shows that the two latter enzyme peaks are more char-

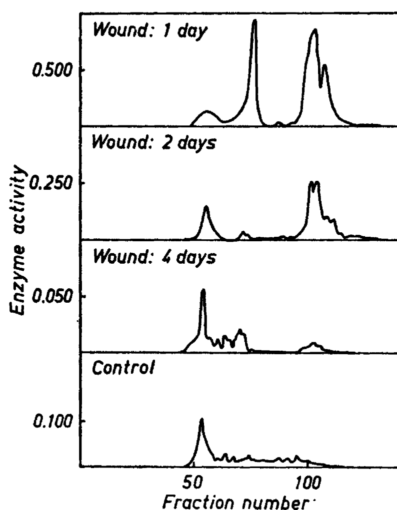


*Fig. 2.* Fractionation of normal and wound tissue enzymes from the supernatant fluid of the tissue homogenate. Substrate: L-valyl-2-naphthylamide; Column: CM-cellulose, 15 × 900 mm; Buffer: 0.01 M tris-HCl, pH 7.15; Temperature: 5°C; Flow rate: 6 ml/h; Hydrostatic pressure: 100 cm $H_2O$; Sample: 2 ml of the supernatant fluid of the homogenate; Fraction volume: 2 ml; For further details as to the used adsorbent, see text.

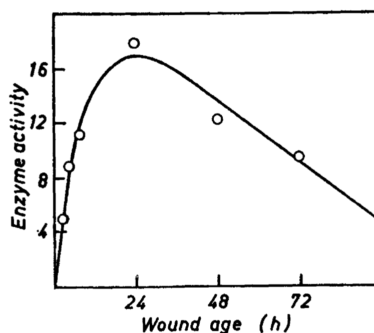


*Fig. 3.* Changes in enzyme activity by L-valyl-2-naphthylamide in rat skin wounds as a function of time. The activities are expressed as the ratio of the activity of the wound to that of the control wound.

acteristic of the wound tissue, and that the second enzyme peak is so labile that it was not possible to distinguish it in the chromatograms of two and four day-old wounds.

Because L-valyl-2-naphthylamide was the substrate whose hydrolysis was most increased in the regenerating wound tissue, it was interesting to study changes of the corresponding enzyme activity as a function of the age of the wound. Fig. 3 shows the results from such an experiment. The action on L-valyl-2-naphthylamide increased up to about 24 h, after which it began to fall.

In order to obtain more evidence for the differences of wound enzymes compared to the normal tissue enzymes, attempts were made to fractionate them by ammonium sulphate precipitation. Fig. 4 shows the results. At least two points are apparent. Firstly, the action on L-valyl-2-naphthylamide was clearly higher in the wound tissue than in the normal tissue (the protein concentration was the same in both preparations). Secondly, the enzymes hydrolysing that substrate in the wound tissue needed a higher concentration of ammonium sulphate to be precipitated totally.

Fractionation through Sephadex G-200 columns shows that the protein concentration in the wound is also increased during the regeneration process. Fig. 5 shows the amount of protein in different Sephadex fractions of a typical two day-old wound tissue and of normal tissue. Two main protein peaks were obtained, the latter one being larger in the wound tissue than in the normal one.

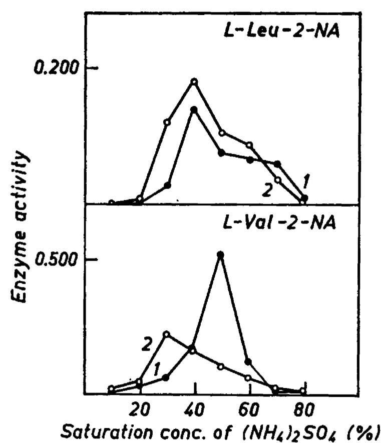


*Fig. 4.* Sedimentation of the enzymes hydrolysing 2-naphthylamides of L-leucine and L-valine from the supernatant fluid of the tissue homogenate using $(NH_4)_2SO_4$. The precipitate obtained from 2 ml of the starting material at the $(NH_4)_2SO_4$ concentration was dissolved in 0.5 ml 0.1 M tris-HCl buffer, pH 7.15. 1 = wound; 2 = control.

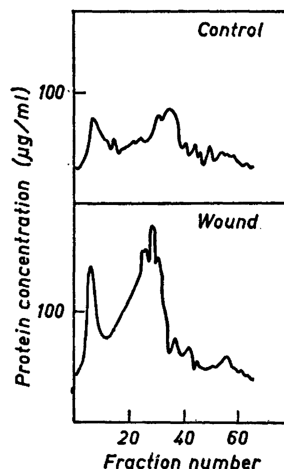


*Fig. 5.* Fractionation of proteins from the supernatant fluid of the tissue homogenates as estimated by Folin-Ciocalteu method. Column: Sephadex G-200, 15 × 900 mm; Buffer: 0.05 M tris-HCl buffer, pH 7.15; Temperature: 8°C; Flow rate: 6 ml/h; Hydrostatic pressure: 20–25 cm $H_2O$; Sample: 2 ml; Fraction volume: 2 ml.

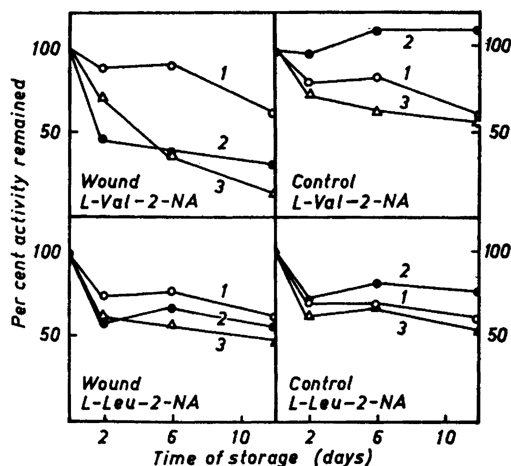


*Fig. 6.* Preservation of aminopeptidase-like enzyme activities of the supernatant fluid of the homogenate. The ordinate is expressed as the enzyme activity remaining after the time involved. 1 = −20°C; 2 = +24°C; 3 = +4°C.

Fig. 6 gives the results from experiments in which the supernatant fluid of the homogenate obtained from two dayold wounds and from normal tissue was exposed to different temperatures in 0.1 M tris-HCl buffer, pH 7.15. The activity of the preparation was tested at intervals of some days by using 2-naphthylamides of L-valine and L-leucine. In both tissues the enzyme(s) hydrolysing L-leucyl-2-naphthylamide were preserved in quite a similar way at the three temperatures tested, and the corresponding enzyme activities were lowered just a little. On the other hand, in the wound tissue the action on L-valyl-2-naphthylamide was rapidly destroyed during the storage, whereas that of the normal tissue was nearly the same as at the beginning of the experiment. Thus, the wound tissue enzymes hydrolysing L-valyl-2-naphthylamide seem to be more labile than those of the control tissue.

## DISCUSSION

Weimar and Haraguchi [9] have observed that there is normally no succinic acid dehydrogenase and 5′-nucleotidase in the corneal connective tissue of the rat. In the lag-phase of the wound-healing process these enzymes are either formed or they are activated in some way. Consequently, it is possible that some of the aminopeptidase-like enzymes would not have been revealed if the test material had consisted of intact skin only.

The infliction of a wound produces serious changes at the cellular level. In the present paper several facts can be listed to show that a biosynthesis of new enzyme proteins, specific for the wound tissue, takes place in the wound.

a) The amount of some proteins in the wound tissue is higher than in the normal tissue. This is shown in Fig. 5. The latter protein peak in the chromatogram was always higher than that of the normal tissue. In a following

paper it will be shown that this second peak obtained with wound tissue always showed increased enzyme activities. It could be suggested that the increase in the protein concentration is partly due to the biosynthesis of new enzyme molecules.

b) The use of control wounds eliminated the possibility that the increased enzyme activities in the wound are due to bleeding.

c) Fig. 4 shows that in the wound tissue there exist enzyme(s) hydrolysing L-valyl-2-naphthylamide, which need a higher ammonium sulphate concentration to be totally precipitated than the normal tissue enzyme(s) hydrolysing that substrate. Thus, it is evident that the wound tissue contains protein molecules of different structure and nature as compared with the normal skin enzymes.

d) The enzymes in the wound tissue hydrolysing L-valyl-2-naphthylamide are more labile during the storage than those in the normal tissue. This indicates the presence of specific wound tissue aminopeptidase-like enzymes in the used test material.

e) The CM-cellulose chromatogram in Fig. 2 clearly shows the existence of specific wound tissue aminopeptidase-like enzymes not present in the normal tissue preparations.

It has been shown earlier by Raekallio and Mäkinen [6] that the enzyme activity demonstrable by L-leucyl-2-naphthylamide in the wound tissue increases at least up to several days. On the other hand, the present studies demonstrate that the hydrolysis rate of L-valyl-2-naphthylamide by wound enzymes begins to decrease from about 24 h. This suggests the existence of differences among the wound tissue enzymes.

The preliminary fractionation procedures presented in this paper show that the separation of wound enzymes hydrolysing 2-naphthylamides of various amino acids is possible using, *e.g.*, ammonium sulphate precipitation and CM-cellulose chromatography.

*Acknowledgement.* This investigation was supported by a research grant from the *Sigrid Jusélius Foundation* and from the *Finnish Medical Council.*

## REFERENCES

1. Monis, B. *Am. J. Pathol.* **42** (1963) 301.
2. Raekallio, J. *Nature* **188** (1960) 234.
3. Raekallio, J. *Ann. Med. Exptl. Biol. Fenniae (Helsinki)* **39** *Suppl.* **6** (1961).
4. Raekallio, J. *Exptl. Molec. Pathol.* **4** (1965) 303.
5. Raekallio, J. *Die Alterbestimmung mechanisch bedingter Hautwunden mit enzymhistochemischen Methoden,* Verlag Schmidt-Römhild, Lübeck 1965.
6. Raekallio, J. and Mäkinen, P.-L. *Nature* **213** (1967) 1037.
7. Layne, E. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology,* Academic, New York 1958, Vol. III, p. 447.
8. Peterson, E. A. and Sober, H. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology,* Academic, New York 1962, Vol. V.
9. Weimar, V. L. and Haraguchi, H. *Invest. Ophtalmol.* **4** (1965) 853.

Received December 13, 1966.