# Characterization of Elastase-like Enzymes in Various Blistering Diseases

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Oikarinen A, Kiistala U, Uitto J. Characterization of elastase-like enzymes in various blistering diseases. Acta Derm Venereol (Stockh) 1986; 66: 1–5.

Blister fluid samples were collected from suction induced control blisters and from spontaneous blisters from various blistering diseases for the measurement of elastase-like enzyme activities using synthetic succinyl-L-(alanyl)$_3$-paranitroanilide (SAPNA) and alanyl paranitroanilide (ala-PNA) as the substrates. The blister fluids derived from bullous pemphigoid, pyoderma or epidermolysis bullosa dystroficans lesions contained higher levels of elastase-like enzyme activities than burn blisters or fresh suction blisters. The gel filtration studies using Sepharose CL-4B chromatography revealed two major peaks of SAPNA hydrolysing enzyme activity in burn blister and in bullous pemphigoid blister. The first peak eluted in void volume, and the second peak had an apparent molecular weight of $2.5 \times 10^5$ daltons. The results indicate that various proteinases are present in blisters fluids and that they may participate in the blister formation by degrading structural components of the basement membrane zone and the dermis. *Key words: Proteolytic enzymes; Bullous diseases.* (Received May 28, 1985.)

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In a recent study we demonstrated the activity of elastase-like enzyme in blistering diseases (1). In pemphigoid blisters, we found a neutral metalloenzyme capable of hydrolysing a synthetic substrate succinyl-L-(alanyl)$_3$-paranitroanilide. The origin and significance of this enzyme has not been studied in detail. A similar elastase-like enzyme has been thoroughly characterized from sera of a patient with cutis laxa and pulmonary emphysema (2).

In the present study we have examined elastase-like enzymes in various blistering diseases in more detail using two different small molecular weight substrates. In addition, we examined the blister samples for the presence of protease inhibitors, especially α2-macroglobulin, in order to elucidate the balance between proteolytic enzymes and their inhibitors in blistering diseases.

## PATIENTS AND METHODS

Blister fluid samples were taken from five patients with bullous pemphigoid (BP), from three burn blisters, as well as from patients with pyoderma and recessive epidermolysis bullosa dystroficans (REBD), one case each. As a control, suction blisters (3), were induced on the abdominal skin of seven healthy controls, and blister specimens were taken immediately after blister formation (0-h samples) as well as after 24, 48 and 72 hours. The samples were immediately frozen and stored at −20°C until assayed.

Elastase-like enzyme activity was assayed by using synthetic succinyl-(L-alanyl)$_3$-paranitroanilide (SAPNA; Sigma Chemical Co., St. Louis, MO) as substrate (4). For the assay, 10–40 µl aliquots of blister samples, were incubated with SAPNA in 50 mM Tris-HCl, pH 7.8, in a final volume of 1 ml for various time periods up to 24 h, at 37°C. The rection was monitored by the change of absorbance at 410 nm, and the activity was expressed as hydrolysis of SAPNA, nmol/h/ml blister fluid. Control tubes without added enzyme were included in all incubations. Hydrolytic activity in blister fluids was also assayed using a synthetic alanyl-paranitroanilide (ala-PNA; Vega Biochemicals, Tucson, AZ) as the substrate under similar conditions that was used with SAPNA (5). The reaction was monitored as

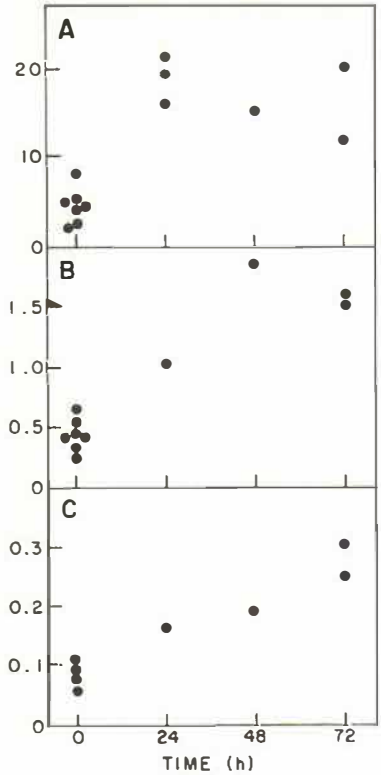


*Fig. 1.* The SAPNA and ala-PNA hydrolyzing activities and α2-macroglobulin in blister fluids as a function of time (A) SAPNA hydrolyzing activity, (B) Ala-PNA hydrolyzing activity, and (C) α2-macroglobulin. The 0-point represents the time point immediately after blister formation. Each dot represents the mean value of duplicate assays from one individual at the time point indicated.

the absorbance change at 410 nm, and the activity was expressed as hydrolysis of ala-PNA, μmol/h/ml.

*Other assays*

For gel filtration studies, blister fluid samples were chromatographed on a Sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) column, 2.5×90 cm, equilibrated and eluted with 1 M NaCl, 5 mM $CaCl_2$, and 50 mM tris-HCl pH 7.6 at 4°C. Two ml fractions were collected and aliquots were used for

Table I. *Hydrolysis of SAPNA and ala-PNA by blister fluids and the concentrations of protein and α2-macroglobulin*

| Group | No. | Hydrolysis of SAPNA (nmol/h/ml) | Hydrolysis of ala-PNA (μmol/h/ml) | Protein (mg/ml) | α2-macroglobulin (mg/ml) |
|---|---|---|---|---|---|
| Bullous pemphigoid | 5 | 21.2±8[a] | 1.42±0.29[a] ($n$=3) | 36.8±5.3[b] | 0.23±0.07[c] |
| Burn blisters | 3 | 7.6±3.7 | 0.89 ($n$=2) | 43.9 ($n$=2) | 0.13±0.09 |
| Pyoderma manum | 1 | 19.4 | 3.80 | 56.1 | 0.30 |
| Recessive epidermolysis bullosa dystroficans | 1 | 30.8 | 0.55 | 31.4 | 0.28 |
| Control suction blisters (0-h) | 5 | 4.4±2.1 | 0.43±0.14 | 28.4±4.3 | 0.09±0.003 |

[a] Statistically different from the control suction blisters; $p<0.001$.
[b] Statistically different from the control suction blisters; $p<0.05$.
[c] Statistically different from the control suction blisters; $p<0.01$.

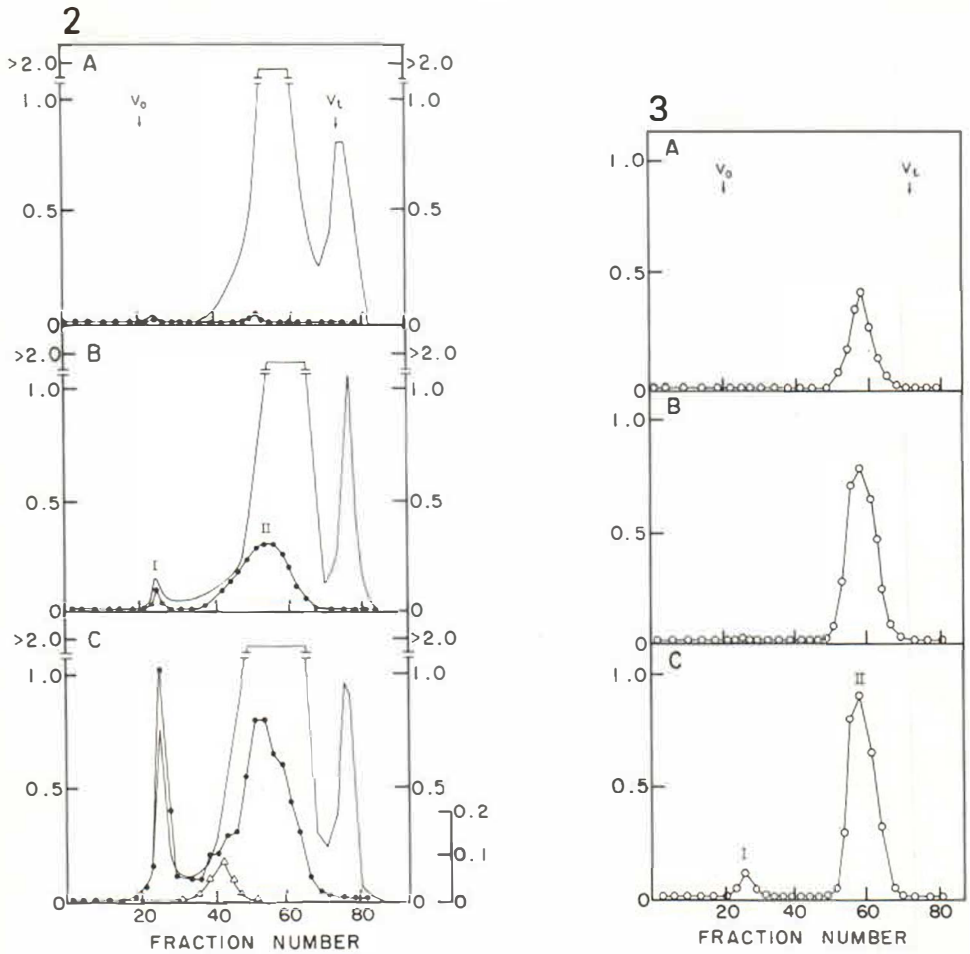


*Fig. 2.* Elution profile of protein and SAPNA hydrolyzing enzyme activity in suction blister (A), burn blister (B) and bullous pemphigoid blister (C). 2.0 ml of blister fluid were chromatographed on a Sepharose CL-4B column, and 2 ml fractions were collected. The fractions were assayed for protein by absorbance at 280 nm and for enzyme activity using SAPNA as the substrate. α2-macroglobulin was assayed by radial immunodiffusion from fractions after chromatography with BP blister fluid. The void volume (Vo) and total volume (VT) of the column were determined by Dextran Blue 2000 and [$^3$H] $H_2O$ elution, respectively. ●—●, enzyme activity by absorbance at 410 nm; ——, protein, absorbance at 280 nm; △—△, α2-macroglobulin.

*Fig. 3.* Elution profile of ala-PNA hydrolyzing enzyme activity in suction blister (A), burn blister (B) and in bullous pemphigoid blister (C). The samples and the conditions were the same as in Fig. 2. The enzyme activity was assayed using ala-PNA as the substrate.

enzyme assays. The protein concentration was monitored by absorbance at 280 nm. The void volume (Vo) of the column was determined by using Dextran Blue 2000 (Pharmacia) and the total volume (Vt) was estimated from the elution of [$^3$H]$H_2O$ (Amersham). The column was calibrated with thyroglobulin (m.w. 689000), ferritin (m.w. 440000), catalase (m.w. 232000), and aldolase (m.w. 158000), all from Pharmacia.

α2-Macroglobulin concentration in blister samples were determined by radial immunodiffusion, using 0.6% agarose gels containing 25 mM tris-HCl, pH 7.4, and 112 ng/ml rabbit antihuman α2-macroglobulin (Calbiochem-Behring) (6). The protein concentration in blister samples was assayed by a method of protein-dye binding (7).

## RESULTS

In bullous pemphigoid blisters, SAPNA and ala-PNA hydrolyzing activities were significantly higher than in control suction blisters. In burn blisters, the enzyme activities were on the same level as in control blisters (Table I). In one pyoderma blister, ala-PNA activity was almost ten-fold increased as compared to control values, and in REBD blisters SAPNA hydrolyzing activity was about seven times higher than in controls. The protein levels were increased in blister fluids derived from patients compared to controls. α-2 Macroglobulin was increased in BP, pyoderma and REBD blisters when compared to controls (Table I).

Since the age of blisters could affect the enzyme and α2-macroglobulin values, these parameters were followed in control suction blisters by taking samples from blisters of 0, 24 h, 48 h and 72 h of age. A marked increase was noted in SAPNA activity already after 24 h (Fig. 1 A). Similarity ala-PNA activity and α2-macroglobulin increased in older suction blisters, the values being about three fold in 72 hour blisters compared to 0-h blisters (Fig. 1 B, C).

### *Gel filtration studies*

The blister samples (2 ml in each) from 0-h control suction blisters, from burn blister and from bullous pemphigoid blister were analysed by gel filtration on a Sepharose CL-4B column. The fractions were then assayed for SAPNA and ala-PNA activities, and the protein concentration was monitored by absorbance at 280 nm. The protein eluted in three peaks in all blister samples chromatographed (Fig. 2 A–C), the first peak eluting close to void volume (Vo), and the last peak in the total volume (Vt) of the column. Most of the proteins eluted between the fractions of 40–65. It should be noted that the first peak eluting in void volume was very small in suction blister fluid (Fig. 2 A) and in burn blister (Fig. 2 B).

The SAPNA hydrolyzing activity eluted in two peaks in burn blister (Fig. 2 B) and in BP blister (Fig. 2 C). In suction blister the activity was very small, and most of the activity eluted in fraction 50–54 (Fig. 2 A). The first peak in burn blister and BP blister eluted in void volume of the column indicating that the molecular weight was in excess of $10^7$ daltons. The second peak eluted 40–65, the peak fraction being in 52–54, which corresponds to a molecular weight of $2.5 \times 10^5$.

α-2-macroglobulin eluted between fractions 35–45 in BP blister (Fig. 2 C), indicating that most of SAPNA activity was not associated with α2-macroglobulin.

Most of the ala-PNA hydrolyzing activity eluted between fractions 50–70, the peak vlaues being in fractions 58–60 (Fig. 3). Small activity was noted in void volume in BP blister (Fig. 3 C) and in burn blister (Fig. 3 B).

## DISCUSSION

The results of the present study indicate that SAPNA and ala-PNA hydrolyzing enzymes are different proteins. This is confirmed by elution profiles with gel filtration, in which enzyme peaks were eluted on different fractions. Further, our preliminary studies have shown that granulocyte or pancreatic elastase cannot utilize ala-PNA as the substrate, whereas these enzymes readily hydrolyze SAPNA (Oikarinen A, unpublished).

The enzyme activities in blister fluids from bullous pemphigoid, pyoderma and epidermolysis bullosa blisters were clearly higher than in 0-h control suction blisters. In older suction blisters enzyme activities increased markedly in comparison to fresh blisters. This indicates that enzymes are released to blister fluid locally or that they diffuse from plasma. The plasma levels of elastase-like enzymes were on the same level in bullous

pemphigoid patients as in control subjects (not shown). Thus the increased elastase activities noted in BP plasma may not be derived only from plasma, but seem to be released locally. Since inflammatory cells migrate to suction blisters (8), it is possible that part of the enzymes is derived from these cells (9). The possible source for SAPNA activity could be the fibroblastic cells (10), since fibroblasts secrete neutral elastase-like enzyme hydrolyzing SAPNA. Previously proteinases using various substrates such as casein and hemoglobin, have revealed the hydrolyzing enzyme activities in spontaneous blisters of blistering diseases. The activities varied markedly from case to case (11). It was suggested that part of enzymes were derived locally from the skin.

The balance between proteolytic enzymes and inhibitors was studied in the present study by assaying α2-macroglobulin. Highest values were found in pyoderma and BP blisters. However, even in these the values were only about 20–30% of corresponding sera values. Interestingly in control blisters α2-macroglobulin levels were increased in older blisters up to three-fold probably by diffusion from sera.

In conclusion the results thus indicate that blister fluids from various blistering diseases contain proteolytic enzymes, which may participate in the degradation of the components of basement membrane zone and the dermis (12).

## ACKNOWLEDGEMENTS

This study was supported in part by the Medical Research Council of the Academy of Finland and by grants AM-28450 and GM-28833 from United Public Health Service, National Institutes of Health.

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