

Dispase, a Neutral Protease From *Bacillus Polymyxa*, Is a Powerful Fibronectinase and Type IV Collagenase

Kurt S. Stenn, M.D., Richard Link, Gisela Moellmann, Ph.D., Joseph Madri, M.D., Ph.D., and Elizabeth Kuklinska, M.S.

Departments of Dermatology and Pathology, Yale University School of Medicine, New Haven, Connecticut, U.S.A.

Dispase, a neutral protease isolated from culture filtrates of *Bacillus polymyxa*, has proven to be a rapid, effective, but gentle agent for separating intact epidermis from the dermis and intact epithelial sheets in culture from the substratum. In both cases it effects separation by cleaving the basement membrane zone region while preserving the viability of the epithelial cells. Because it is not known what or where in the basement membrane zone Dispase cleaves, we set up studies to define its substrate specificity. Using purified basement membrane components and sodium dodecyl sulfate-polyacrylamide gel electrophoresis we show that Dispase cleaves fibronectin and type IV collagen, but not laminin,

type V collagen, serum albumin, or transferrin. The action of Dispase on collagen appears to be selective for type IV collagen in that several stable degradation products are formed, whereas the enzyme degrades type I collagen only minimally. In newborn human skin, as seen by electron microscopy, Dispase removes the lamina densa, rich in type IV collagen, but preserves the anchoring fibrils (structures known to contain type VII collagen) and the epidermal cells. Because its action is so selective, it suggests that Dispase can serve as a powerful tool for dissecting epithelial-mesenchymal interactions. *J Invest Dermatol* 93:287-290, 1989

Earlier studies have demonstrated that Dispase, a neutral protease from *Bacillus polymyxa*, cleaves the basement membrane zone (BMZ) of the skin, sharply separating the epidermis from the dermis [1]. What in the BMZ is cleaved has not been further characterized. To define the substrate specificity of Dispase we have studied its action on various purified components of the BMZ by polyacrylamide gel (PAGE) electrophoresis and its site of cleavage within the BMZ of newborn skin by electron microscopy. This study indicates that type IV collagen, fibronectin, and lamina densa of skin are particularly sensitive, whereas laminin, type V collagen, and anchoring fibrils, which contain type VII collagen [2], are resistant to this enzyme.

MATERIALS AND METHODS

Reagents All chemicals were of reagent grade or better; water was single or double distilled. Dispase, type II from *Bacillus polymyxa*, was obtained from Boehringer-Mannheim (Mannheim, F.R.G.), type I collagen (Vitrogen) from Collagen Corporation (Palo Alto, CA), and bovine serum albumin, collagenase (from *Clostridium histolyticum*), and transferrin from Sigma Chem. Co. (St Louis, MO).

Other proteins were prepared as previously described: type IV [3], type V collagen [4], laminin [5], and plasma fibronectin [6].

Electrophoresis Sodium dodecyl sulfate (SDS)-PAGE electrophoresis, under reducing conditions, was used in all experiments after the method of Laemmli [7] with 6% or 12.5% acrylamide gels. Protein bands were visualized by Coomassie Blue staining. Protein transblotting to nitrocellulose filters was performed as previously described [8]. Polyclonal antitype IV collagen antibody was prepared, purified, and characterized as described by Roll et al [3]. The secondary antibody was peroxidase-labeled (Cappel, Malvern, PA) goat antirabbit and used at a dilution of 1:2000.

Enzyme Reactions The test proteins were incubated with Dispase or clostridial collagenase at a substrate: enzyme concentration ratio of 10:1 in calcium-magnesium free (CMF) Tyrodes solution or with an equivalent volume of buffer alone for 15-18 h at 4°C. All enzyme reactions were stopped by the addition of sample buffer and heat [7].

Electron Microscopy Newborn foreskin was cleaned of subcutaneous tissue, laid flat onto paper towels (dermis side down), and incubated in Dispase (10 mg/ml, CMF Tyrodes, 4°C, 6 h). The specimens were fixed in aldehyde overnight at 4°C, as described previously [9]. Subsequently, they were osmicated in the presence of potassium ferrocyanide (1.5%) and embedded in epoxy resin. Ultrathin sections were stained with uranyl and lead ions.

RESULTS AND DISCUSSION

Dispase, a neutral protease crystallized from the culture filtrate of *Bacillus polymyxa* [10], is active in serum but inactivated by metal-binding reagents (e.g., EDTA) [11]. Electrophoresis of a commercial Dispase preparation reveals a single protein component of Mapp 41 kD (Fig 1). In biologic research, Dispase has proven to be very useful to prepare epidermal sheets from intact skin as well as from confluent epidermal cell cultures [1]. Treatment with Dispase causes separation of the epidermis from the dermis at the BMZ,

Manuscript received December 16, 1988; accepted for publication January 27, 1989.

This work was supported in part by a grant from Chemex Pharmaceuticals, Inc., and NIH grants HL 28373 (JAM), CA 04679, and AM 25252 (AB Lerner).

Reprint requests to: K.S. Stenn, M.D., Department of Dermatology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

Abbreviations:

BMZ: basement membrane zone

CMF Tyrodes: calcium-magnesium free Tyrodes solution

Mapp: approximate molecular weight

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

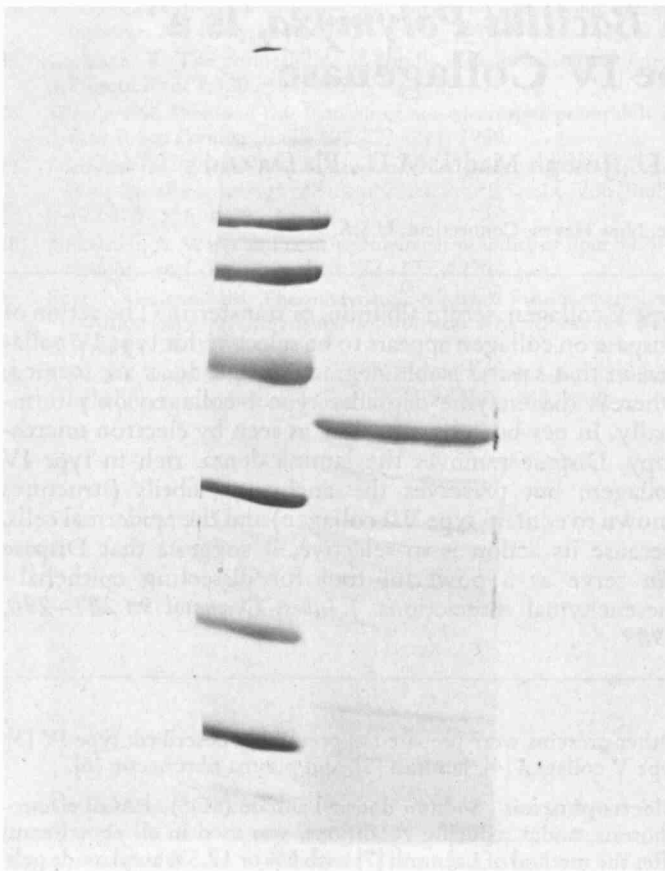


Figure 1. SDS-PAGE electrophoresis of commercial Dispase (Dispase II from *Bacillus polymyxa*, Boehringer-Mannheim) preparation. Acrylamide gels (12.5%) were run using the Laemmli [7] system, and mercaptoethanol reduction. The left lane contains protein molecular weight markers of 94, 67, 43, 30, 20, and 14.4 kD from top to bottom, respectively. Dispase migrates as a thin band with Mapp of 41 kD (arrowhead). The Dispase was heavily loaded with 600 μ g of protein

while concomitantly preserving epidermal cell viability and intercellular attachments. Moreover, in contrast to trypsin, it effects the separation of the epithelial sheet without dissociating the individual keratinocytes.

The protein specificity of Dispase within the BMZ was investigated using purified BMZ components and SDS-PAGE electrophoresis (Fig 2). The conditions of incubation were identical to those routinely used in preparing epidermal sheets from whole skin (i.e., 15–18 h of incubation in the cold at 4°C in CMF Tyrodes).

Within the limits of this approach the electrophoretic pattern shows that Dispase is not a broad-spectrum protease as it produces no apparent effect on the mobility of serum albumin, transferrin, laminin, or type V collagen (Fig 2). Some degradation of type I collagen was observed, but the major bands remained intact. In contrast, the major fibronectin doublet was completely degraded, giving rise to five stable derivatives.

Dispase completely degraded the beta and gamma aggregation forms (and higher oligomers in the stacking gel) of type IV collagen and showed a greater proteolytic effect on the alpha-2 subunit than on the alpha-1 subunit. Two prominent and stable degradation products were formed: a densely staining doublet of Mapp 105 kD and a lighter staining, more diffuse band of Mapp 80 kD. In reduced type IV collagen samples, which have greatly diminished oligomeric forms, incubation with Dispase was also found to degrade the alpha-1 and alpha-2 subunits, producing the same stable 105- and 80-kD degradation products (data not shown). To test if the 105-kD product actually arose from type IV collagen, the band was transblotted and stained with a rabbit polyclonal antibody to type IV

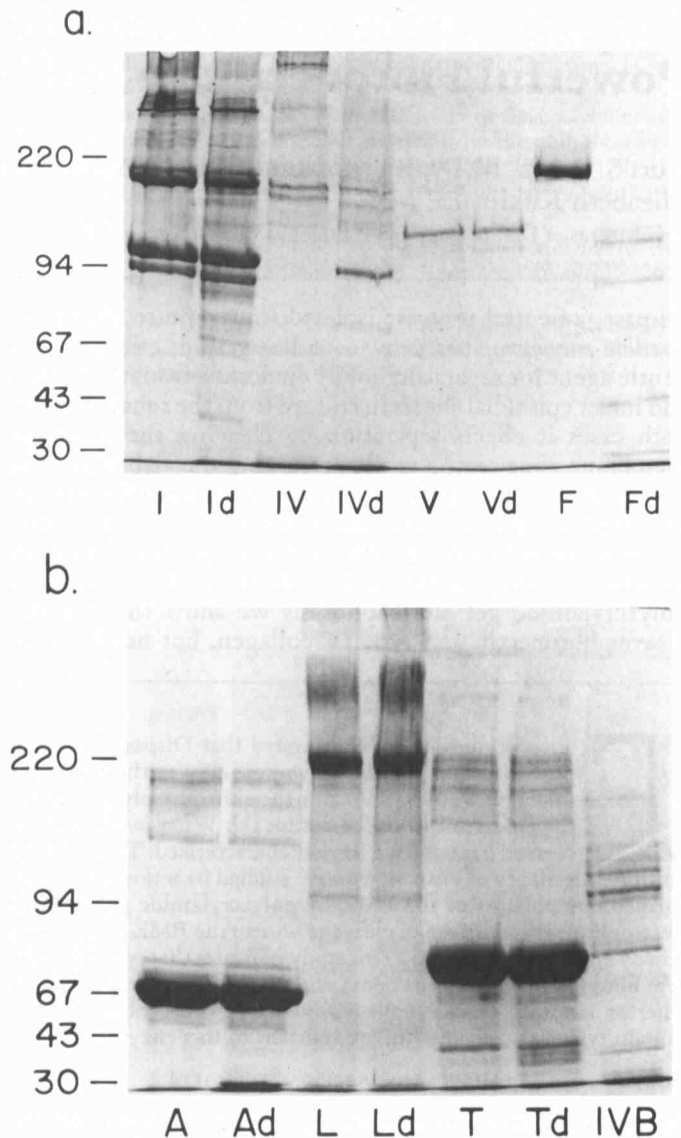


Figure 2. SDS-PAGE electrophoresis patterns (under reducing conditions) of Dispase-exposed purified protein preparations. In (a) and (b) identical conditions were used. Mobility of the molecular weight markers is indicated in kD on the left side of each figure. The lanes are given in pairs with the left side of each pair representing the control (incubated with buffer alone) and the right side a preparation incubated with Dispase(d). The following protein labels are used: I (85 μ g, type I collagen), IV (30 μ g, type IV collagen), V (20 μ g, type V collagen), F (20 μ g, human plasma fibronectin), A (30 μ g, bovine serum albumin), L (15 μ g, laminin), and T (30 μ g, transferrin). The comparative effect of bacterial collagenase (10:1, substrate: enzyme ratio, from *Clostridium histolyticum*) on type IV collagen is shown as the last lane of (b), labeled IVB; the degradation products resulting from this enzyme differ markedly from those wrought by Dispase.

collagen. As shown in Figure 3 all the degradation products were stained with this antibody. Chemical evidence that the 105-kD product is a collagen was obtained after isolation of this derivative by the transblot technique of Matsudaira [12]. By amino acid analysis it contains 33% glycine and 8% proline. Type IV collagen exposed to Dispase at 37°C for 2 h showed an identical degradation pattern (data not shown), supporting the conclusion that the collagenolytic specificity observed is a prominent characteristic of this enzyme preparation and not a minor reaction apparent only at 4°C. That the action of Dispase differs from clostridial collagenase is shown (Fig 2b, lane IVB).

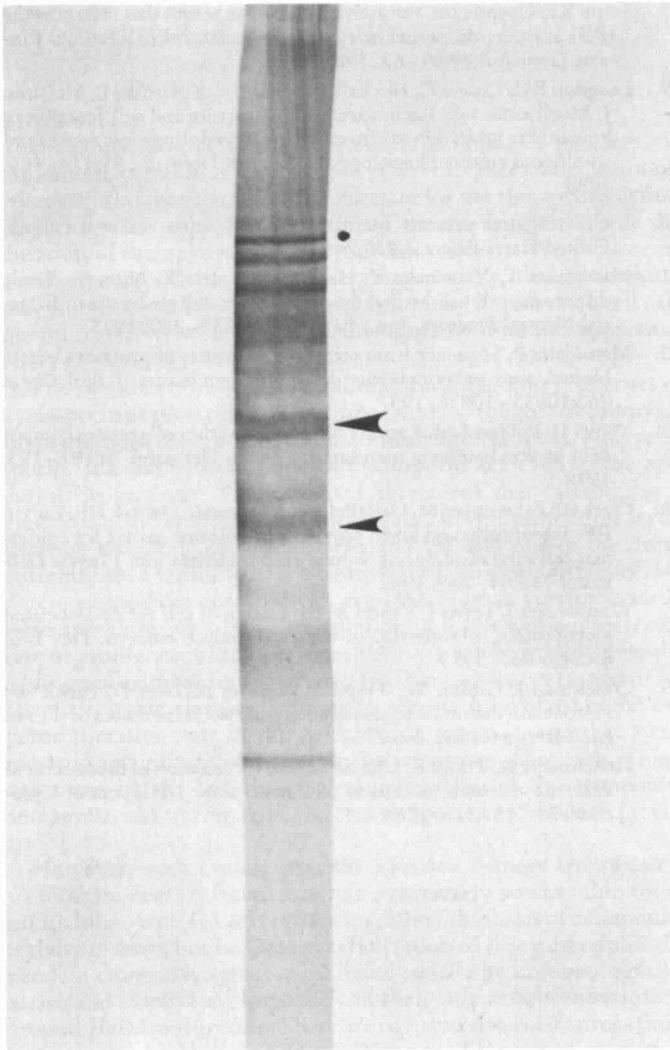


Figure 3. Antitype IV collagen staining of Dispace-hydrolyzed type IV collagen. Under the same conditions as described in Figure 2, Dispace-hydrolyzed type IV collagen was electrophoresed, transblotted to nitrocellulose, and stained as previously reported [8]. The antitype IV collagen antibody stain reveals that all the hydrolyzed products are antigenically type IV collagen, including the prominent 105-kD product (*large arrowhead*) and the more diffuse 80-kD product (*small arrowhead*). The dot marks the alpha-1 chain.

If Dispace acts on type IV collagen, then its action within the BMZ should localize to the lamina densa where the majority of type IV collagen is found [13]. Untrastructural studies bear out this conclusion. As seen in Figure 4, Dispace removes the lamina densa but preserves epithelial cell morphology. The specificity of this collagenolytic activity is for type IV collagen, underscored by the preservation of type VII collagen [2], as manifest by the intact anchoring fibrils projecting into the base of the Dispace-induced blister (Fig 4d).

That Dispace affects predominantly the larger aggregation forms of type IV collagen is interesting and may play a significant role in its action on tissues, i.e., the cross-link regions of the molecule may be the most Dispace-sensitive. Work in progress on the peptide specificity of Dispace should yield some insight into the specific chemical mechanism of action of this enzyme on BMZ structures.

The correlation between the fibronectinase activity of Dispace and the tissue cleavage site is more difficult to explain. Although fibronectin is more apparent in the epidermal BMZ during wound repair and development [14,15], it is reported to be present in small amounts in the lamina lucida and about the anchoring fibrils of adult

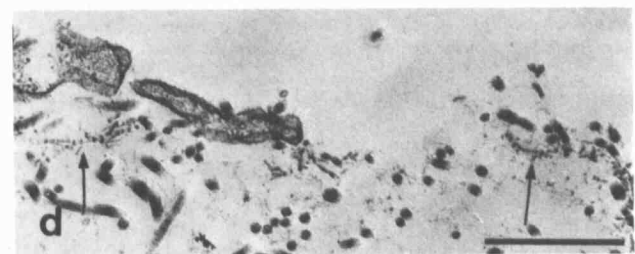
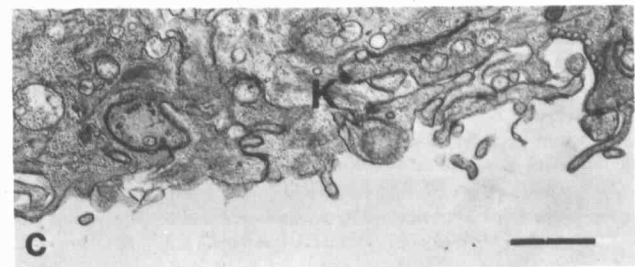
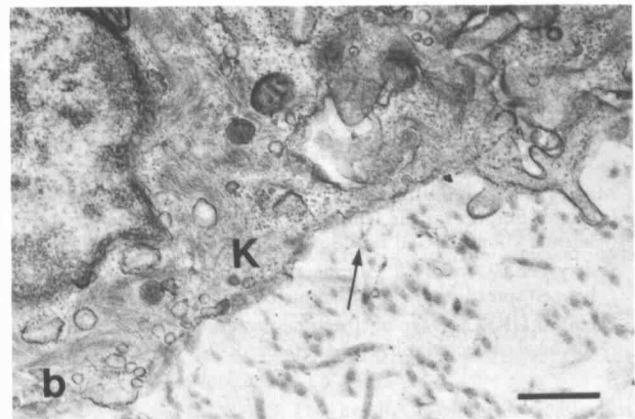
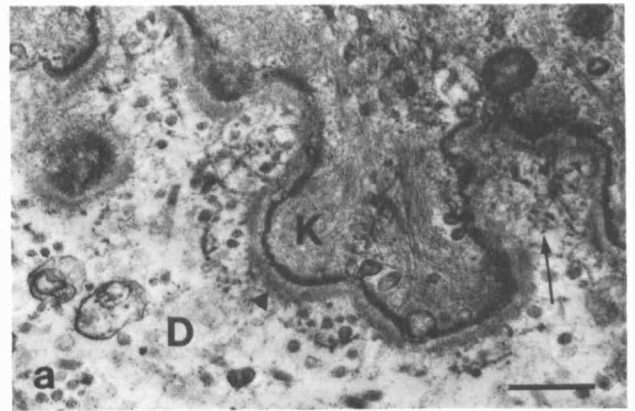


Figure 4. Electron micrographs of the basement membrane zone in the human newborn foreskin before and after exposure to Dispace. *a*: Control specimen (time 0) shows an intact BMZ (*K*, basal keratinocyte of epidermis; *D*, dermis; *arrows*, anchoring fibrils; *arrowheads*, lamina densa of basal lamina). *b-d*: Experimental specimens (6 h incubation with Dispace), derived from adjacent ultrathin sections, show absence of the basal lamina in the BMZ, the epidermis and dermis still adhering in spots (*b*) and separated elsewhere (*c,d*); preservation of anchoring fibrils and their association with the dermal component of split skin (*d* vs *c*); and optimal preservation of basal keratinocytes (*K*, *b*, and *c*). Micrographs *c* and *d* originate from the same electron-negative and represent, respectively, the top (epidermis) and bottom (dermis) of a zone of detachment approximately 2 μm wide. (Bars = 0.5 μm .)

healthy skin [16,17]. Whether this minimal amount of BMZ fibronectin is structurally inherent or simply a contaminant from serum has not been resolved. That fibronectin may play a role in promoting cell adhesion to type IV collagen may, however, be relevant to the observed suppression of follicular epithelial growth after Dispase exposure (Link et al, submitted for publication).

Basement membrane phenomena, including epithelial mesenchymal interactions, are very complex. Having tools that specifically dissect these interactions will lead to a deeper understanding of these interactions. Dispase is one such tool.

REFERENCES

- Green H, Kehinde O, Thomas J: Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. *Proc Natl Acad Sci USA* 76:5665-5668, 1979
- Sakai LY, Keene DR, Morris NP, Burgeson RE: Type VII collagen is a major structural component of anchoring fibrils. *J Cell Biol* 103:1577-1586, 1986
- Roll J, Madri JA, Albert J, Furthmayr H: Codistribution of collagen types IV and AB in basement membranes and mesangium of the kidney. *J Cell Biol* 85:597-616, 1980
- Madri JA, Furthmayr H: Isolation and tissue localization of type AB collagen from normal lung parenchyma. *Am J Pathol* 94:323-331, 1980
- Madri JA, Roll J, Furthmayr H, Foidart J-P: Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney. *J Cell Biol* 86:682-687, 1980
- Federgreen W, Stenn KS: Fibronectin (LETS) does not support epithelial cell spreading. *J Invest Dermatol* 75:261-263, 1980
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685, 1970
- Stenn KS: Coepibolin, the activity of human serum that enhances the cell spreading properties of epibolin, associates with albumin. *J Invest Dermatol* 89:59-63, 1987
- Langdon RC, Cuono C, Birchall N, Madri JA, Kuklinska E, McGuire J, Moellmann GE: Reconstitution of structure and cell function in human skin grafts derived from cryopreserved allogeneic dermis and autologous cultured keratinocytes. *J Invest Dermatol* 91:478-485, 1988
- Irie Y: Neutral protease useful for animal tissue and cell culture. United States Patent 3,930,954, 1979
- Matsumura T, Yamanaka T, Hashizume S, Irie Y, Nitta K: Tissue dispersion, cell harvest and fluid suspension culture by use of Bacterial Neutral Protease. *Jpn J Exp Med* 45:377-382, 1975
- Matsudaira P: Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 262:10035-10038, 1987
- Yaoita H, Foidart J-M, Katz S: Localization of the collagenous component in skin basement membrane. *J Invest Dermatol* 70:191-193, 1978
- Clark RAF, Lanigan JM, DellaPelle P, Manseau E, Dvorak HF, Colvin RB: Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. *J Invest Dermatol* 79:264-269, 1982
- Wartiovaara J, Leivo I, Vaheri A: Expression of cell surface-associated glycoprotein, fibronectin, in the early mouse embryo. *Dev Biol* 69:247-257, 1979
- Couchman J, Gibson W, Thom D, Weaver A, Dees D, Parish W: Fibronectin distribution in epithelial and associated tissues of the rat. *Arch Dermatol Res* 266:295-310, 1979
- Fleischmajer R, Timpl R: Ultrastructural localization of fibronectin to different anatomic structures of human skin. *J Histochem Cytochem* 32:315-321, 1984