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Purification and Characterization of Protease Activated by Sulfur Mustard in Normal Human Epidermal Keratinocytes

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ABSTRACT. A membrane-bound protease induced by sulfur mustard in cultured normal human epidermal keratinocytes (NHEK) was purified and partially characterized. Maximum enzyme stimulation occurred at 16 hr after normal human epidermal keratinocytes were exposed to 300 μM sulfur mustard. Purification to homogeneity of the protease was accomplished by Triton X-100 solubilization, ultracentrifugation, and dialysis, followed by ion-exchange chromatography through DEAE-cellulose and finally hydrophobic column chromatography through phenyl Sepharose. Analysis of the purified enzyme by SDS–PAGE revealed a single polypeptide at the 80 kDa region. Further investigation of biochemical properties showed that a synthetic serine-specific Chromozym TRY peptide and the physiological protein laminin were good substrates for this enzyme. Moreover, this enzyme was inhibited mostly by the serine-protease inhibitors leupeptin and di-isopropyl fluorophosphate and not by the cysteine protease inhibitor E-64 or the metalloprotease inhibitor 1,10-phenanthroline (Component H, CH), indicating the serine protease nature of this enzyme. This enzyme had a pH optimum in the range of 7.0 to 8.0. Amino acid sequencing of the purified enzyme revealed that this enzyme belongs to the endopeptidase family (serine protease), and is homologous with a mammalian-type bacterial serine endopeptidase that can preferentially cleave K-X, including K-P. These results suggest that serine-protease stimulation may be one of the mechanisms of mustard-induced skin blister formation, and that some specific serine-protease inhibitors may be useful for the treatment of this sulfur mustard toxicity. BIOCHEM PHARMACOL **56**;4:467–472, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. sulfur mustard; protease; human epidermal keratinocytes; protease purification; laminin

SM§ is a blistering agent that attacks not only the skin but all epithelial tissues that are exposed to it. To date, no effective antidote against SM-induced toxicity has been discovered. This is mostly due to a lack of understanding of its mechanism of action. It has been suggested that protease activation might be responsible for SM-induced skin injury, resulting in cytopathology and tissue destruction [1, 2] leading to inflammation and blister formation. A cytotoxic dose of SM has been shown to induce trypsin-like and thrombin-like protease in human peripheral blood lymphocytes [3], skin cells [4], and other tissues [5]. However, no systematic study has been carried out thus far to isolate, purify, and characterize the protease(s) responsible for mustard-induced pathogenesis in human skin. Moreover, there is a lack of knowledge about the molecular target(s) or the specific physiological substrates of mustard-induced protease, particularly with respect to basement membrane of the dermal–epidermal junction. In view of these facts, we deemed it important to isolate, purify, and characterize the protease stimulated by SM in human skin cells using cultured NHEK. The results might provide valuable information on the mechanism of the blistering response to SM, particularly with respect to the basement membrane components (e.g. laminin), which are mediators of cell attachment to the basement membrane [6]. A preliminary report of this work has been presented [7].

MATERIALS AND METHODS

Materials

NHEK, KBM, supplements for KBM (insulin, bovine pituitary extract, human epidermal growth factor, hydrocortisone, and gentamicin sulfate), and kits for splitting cells (buffer, trypsin-EDTA, and trypsin-neutralizing solution) were purchased from Clonetics. SM (>98% pure) was from the Edgewood Research, Development and Engineering

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§ *Abbreviations:* BAEE, benzoyl arginine ethyl ester; DFP, di-isopropyl fluorophosphate; KBM, Keratinocytes basal medium; NHEK, normal human epidermal keratinocytes; PMSF, phenylmethyl sulfonyl fluoride; SM, [bis(2-chloroethyl sulfide)] sulfur mustard; TCA, trichloroacetic acid; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; and TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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Center. Molecular weight markers, precast SDS–PAGE gels (10–20%), and a silver stain kit were from Bio-Rad. Chromozym TRY (serine-protease specific) substrate (carbobenzoxy-valyl-glycyl-arginine-4-nitranilide acetate) was from Boeringher Mannheim. Azocasein, TCA, DFP, DEAE-cellulose, phenyl Sepharose, and laminin were from the Sigma Chemical Co. Prototypic protease inhibitors were from Molecular Probes. Coomassie plus protein assay reagent was from Pierce.

Cell Culture and SM Treatment

NHEK cultures were initiated in KBM from frozen stock (passage 2) using 0.25×10^6 cells per 150 cm^2 plastic tissue culture flasks. Cells were grown at 37° in a humidified atmosphere of 95% air/5% CO_2 according to the method described by Rhoads *et al.* [8]. Approximately 70–80% confluent cultures were subcultured to passage 3 to be used in the experiments. Treatment of cells with 300 μM SM was carried out according to the method described by Broomfield and Gross [9]. Cells were exposed to this SM concentration because it is considered to be the *in vitro* equivalent to an *in vivo* vesicating dose of SM.

Determination of Protein and Enzyme Activity

Protein concentration was determined by the Coomassie plus protein assay method according to Smith *et al.* [10]. Protease activity in column fractions was assayed according to Cowan *et al.* [3] using 100 μL Chromozym TRY as substrate in 800 μL of buffer (20 mM Tris–Cl buffer, pH 7.4). The total volume of the reaction mixture was 1 mL in which 100 μL of each column fraction was used as the enzyme source. The reaction mixture was incubated for 30 min at 37°, then stopped by 100 μL of 20% TCA, and kept in ice for 30 min, followed by a 30-min centrifugation at 500 *g*. The supernatant was removed, and its absorbance was read at 405 nm. Standard trypsin (500 μL, 100 ng/mL; equivalent to 0.65 BAEE unit) was used instead of purified enzyme in the control tubes, which produced an absorbance of 0.61 at 405 nm. All enzyme activities were expressed in terms of BAEE units. One BAEE unit is equivalent to 76.9 ng standard trypsin.

Enzyme activity was assayed according to Chakrabarti and Banik [11], using azocasein (6%) as substrate. The same protocol (as used for Chromozym TRY substrate) was used except for the determination of absorbance at 366 nm.

Protease Isolation

NHEK cultures (80% confluent) in 150 cm^2 flasks were exposed to 300 μM SM in KBM at room temperature according to approved procedures at the U.S. Army Medical Research Institute of Chemical Defense. The flasks containing SM-exposed cells were first kept inside a chemical hood for 2 hr at room temperature and then were transferred to a 37° incubator with 5% CO_2/95% air for 5–6 hr. Then the SM exposure medium was replaced by fresh KBM, and cells were incubated overnight at 37° inside a CO_2 incubator. The cells were washed twice using 20 mL/flask of PBS at room temperature. The cells were detached by scraping in 0.32 M sucrose containing 2 mM EDTA (20 mL/flask) and homogenized in a glass–teflon homogenizer by 15 up-and-down strokes and centrifuged at 105,000 *g* for 1 hr at 4°. The pellet was homogenized in cold 20 mM Tris–Cl buffer (pH 7.4) containing a mixture of protease inhibitors (E-64, TLCK, TPCK, pepstatin, Component G, PMSF, Component H, and leupeptin, all at 0.1 mg/mL), 1 mM EDTA, 1 mM sodium azide, 5 mM β-mercaptoethanol, and 1.0% Triton X-100. Because mustard-stimulated protease is membrane bound [12], the homogenate was stirred in the homogenization buffer containing 1% Triton X-100 for 2 hr at 4°, and this suspension was subsequently centrifuged for 1 hr at 105,000 *g*. The supernatant obtained was dialyzed overnight at 4° against the same buffer.

Purification of Protease

Only supernatant after Triton X-100 treatment was found to contain proteolytic activity. Eighty milligrams of protein was applied to an ion exchange (DEAE-cellulose) column (2.5 × 40 cm) equilibrated with buffer A (20 mM Tris–Cl, pH 7.4) containing 1 mM EDTA and 1 mM NaN_3. Flow rate was adjusted to 30 mL/hr. The column was washed first with two column volumes of buffer A and then eluted with two column volumes of 0 to 0.5 M NaCl gradient in buffer A. Fractions (5 mL) were collected, and the absorbance of every alternate fraction was read at 280 nm. Fractions containing protein were tested for protease activity using Chromozym TRY as a substrate according to the method described earlier.

Enzymatically active fractions were pooled together, and total protein content was measured by the Coomassie plus method. Pooled fractions were applied onto a hydrophobic column—phenyl Sepharose (1.5 × 20 cm) equilibrated with buffer A. Then the column was washed with two column volumes of buffer A, followed by 1% ethylene glycol in buffer A. We used a gentle elution with 1% ethylene glycol in order to prevent the degradation of the SM-stimulated protease. Flow rate was adjusted to 20 mL/hr. Fractions (3 mL) were collected, and absorbance of every alternate fraction was read at 280 nm. Fractions containing protein were tested for protease activity. Fractions containing protein but no protease (Chromozym TRY substrate) were discarded to enrich eluates with the protease of interest. Only enzymatically active fractions were pooled together and used subsequently to characterize the enzyme.

SDS–PAGE Analysis

Enzymatically active fractions from each step of the purification procedure were lyophilized, and SDS–PAGE was performed as described by Laemmli [13] on 10–20% precast

TABLE 1. Purification of protease from NHEK treated with SM

Purification steps	Total protein (mg)	Specific activity (BAEE unit/mg protein/hr)	Purification (-fold)
(A) Whole cell homogenate	91	33.8	
(B) Supernatant (Triton X-100 treated)	80	37.9	1.12
(C) DEAE-cellulose column eluate	5.5	3475.4	102.8
(D) Phenyl Sepharose column eluate	0.03	9426.8	278.9

NHEK were treated with 300 μM SM. A BAEE unit has been defined in Materials and Methods. Each value is the average of two experiments.

gradient minigel (Bio-Rad) along with protein standards (low molecular weight). Protein bands were visualized by silver staining according to the directions of the manufacturer.

Sequence Analysis of Peptides

Attempts to determine the amino terminal sequence using Edman degradation of the purified protease were unsuccessful. Since greater than 80% of eukaryotic proteins have been reported to have blocked amino-termini that preclude direct amino acid sequencing, the purified protease was digested with endopeptidase Lys-C followed by fractionation of the resulting peptides by reversed-phase HPLC. Six of the most symmetrical absorbance peaks were subjected to MALDI (matrix assisted laser desorption ionization) mass spectrometry, and two were selected for peptide sequencing by the Edman degradation analysis method.

Characterization of Purified Protease

EFFECT OF PH. Purified enzyme (0.5 μg of protein) was incubated in five different buffers (20 mM Tris–Cl, pH: 6.0, 6.5, 7.0, 7.5, and 8.0), and the proteolytic activities were determined using the enzyme assay described above. The optimum pH was determined from a plot of enzyme activity versus pH.

EFFECT OF PROTEASE INHIBITORS. The effects of some prototypic protease inhibitors (e.g. the serine protease inhibitors leupeptin and DFP, the cysteine protease inhibitor E-64, and the metalloprotease inhibitor Component H) on the purified protease were studied. The enzyme (0.5 μg protein) was preincubated at 37° for 5 min with the inhibitor (100 μM) prior to addition of the substrate. Enzyme assay was done by incubation of reaction mixtures at 37° for 30 min as described above.

Evaluation of Proteinaceous Substrates

Various synthetic substrates (e.g. azocasein, Chromozym TRY) as well as a physiological substrate (e.g. laminin) were used to determine the substrate specificity of the purified enzyme. Chromozym TRY and azocasein were tested as substrates using the colorimetric enzyme assay described above. Laminin was tested by SDS–PAGE of incubation mixtures of purified enzyme, substrate, and inhibitors. Prior to electrophoresis, reactions were stopped by freezing the reaction mixture in dry ice. The samples were then lyophilized and dissolved in electrophoresis sample buffer. The samples were run on SDS–PAGE (10–20%), followed by silver staining.

RESULTS

The protease activity was found mainly (97%) in the soluble supernatant fraction after treatment of SM-exposed cells with Triton X-100, whereas all of the SM-induced enzyme activity was found to be associated with the cell membrane in the absence of Triton X-100 (results not shown). The steps for the purification of protease from SM-treated NHEK are summarized in Table 1. The protease was found to be purified 279-fold. The specific activity of the enzyme was increased markedly at each purification step.

The elution profile of Triton X-100-solubilized SM-treated cell protein and protease activity loaded onto the DEAE-cellulose column is shown in Fig. 1. Several peaks of protein were obtained with supernatant from SM-treated cells, whereas one major enzyme peak was eluted. The enzyme fraction eluted from the DEAE-cellulose column was purified further, as described in Materials and Methods, by chromatography on a hydrophobic phenyl-Sepharose column to achieve higher purity. The purity of the enzyme fractions was determined by SDS–PAGE. The molecular weight was assessed relative to the electrophoretic mobilities of known protein molecular weight standards. The purified protease, as visualized by silver staining (Fig. 2), was composed only of an 80-kDa subunit.

A protein data base search (GenBank Accession: J05128) based on alignment of amino acid sequences (Table 2) of two peptide fragments (RISNSTSPTSF and GGGAGT THLNVQWQPSGGV) derived from the purified enzyme showed that this enzyme matches protease I (a mammalian-type bacterial serine endopeptidase) precursor [14].

The pH dependence of the protease was tested between pH 5.0 and 8.0. There was very little activity below pH 6.5. Optimum activity was obtained at pH 7.5 (data not shown), indicating the characteristic of a neutral protease.

The purified protease effectively hydrolyzed the synthetic substrates azocasein and Chromozym TRY, as well as the physiological substrate laminin. The degradation of high molecular weight laminin by the purified protease is shown in Fig. 3. Densitometric scanning data of Fig. 3 showed that

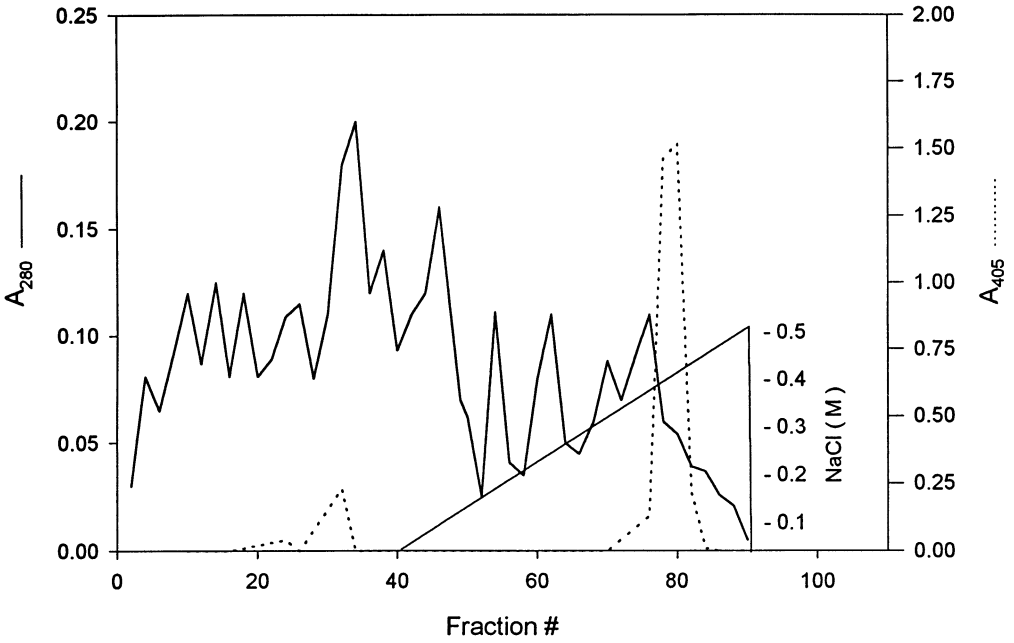

FIG. 1. DEAE-cellulose column separation of SM-induced protease. In the fraction collector, 5 mL/tube was collected, and the absorbance of every alternate fraction was read at 280 nm (protein). Fractions containing protein were tested for protease activity using Chromozym TRY (A_{405}) as a substrate.

proteolysis (53.8% compared with control in Lane L) of laminin (second band shown by arrow) could be inhibited (34.6% proteolysis compared with control) by the serine protease inhibitor leupeptin (100 μM). This proteolysis was not affected (54.8% compared with control) by either the metalloprotease inhibitor 1,10-phenanthroline (also called Component H, CH), or the cysteine protease inhibitor E-64 (61.5% compared with control), indicating that the purified protease was specifically a serine protease.

When Chromozym-TRY was used as a substrate, the serine protease inhibitors leupeptin and DFP at a 100 μM concentration were found to inhibit the purified protease 97 and 89%, respectively (Fig. 4). E-64 (100 μM) and CH (100 μM) caused inhibition to the extent of 30 and 40%, respectively. The effects of E-64 and CH observed in these experiments were probably nonspecific because no inhibition of laminin degradation by the purified protease was seen with either E-64 or CH in the SDS–PAGE analysis (Fig. 3).

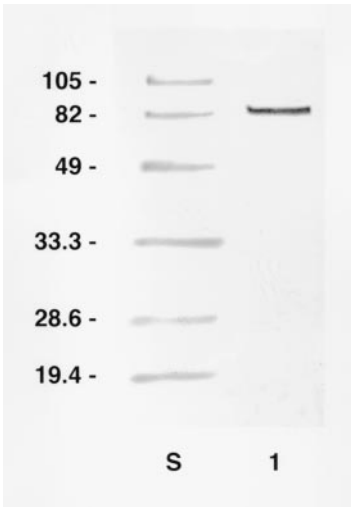

FIG. 2. SDS–PAGE (10–20% gradient) of purified protease from NHEK treated with SM. Silver stain was used to detect protein on the gel. Lane S: low molecular weight Bio-Rad standards containing (from top to bottom) phosphorylase *b*, 105 kDa; bovine serum albumin, 82 kDa; ovalbumin, 49 kDa; carbonic anhydrase, 33.3 kDa; soybean trypsin inhibitor, 28.6 kDa; and lysozyme, 19.4 kDa. Silver stain was used to detect protein on the gel. Lane 1: purified protease (phenyl-Sepharose column eluate).

DISCUSSION

In the present study, we purified mustard-induced protease from NHEK to an apparent homogeneity, as judged by SDS–PAGE and partial amino acid sequence determination. The efficacy of Triton X-100 to release the enzyme from the cell membrane was obvious from our finding that

TABLE 2. Amino acid sequence alignment between bacterial and human protease

Species	Sequence	Sequence similarity (%)
(A) Bacteria	RISNSTSPTSF	
(B) Human	RISNSTSPTSF	100
(C) Bacteria	GGGAGTTHLNVQWQPSGGV	
(D) Human	GGGAGTTHLNVQWQPSGGV	100

Amino acid sequence is arranged from the N terminus to the C terminus.

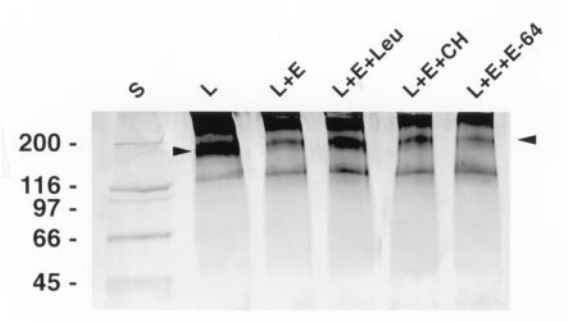

FIG. 3. SDS–PAGE (10–20% gradient) of high molecular weight laminin treated with the purified protease from 300 μM SM-treated NHEK. Silver stain was used to detect protein on the gel. Lane S: high molecular weight Bio-Rad protein standards containing (from top to bottom) myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; and ovalbumin, 45 kDa. Lane L: laminin only, indicated by arrow. Lane L + E: laminin incubated with the purified protease. Lane L + E + Leu, laminin incubated with the purified protease and the serine protease inhibitor leupeptin (100 μM). Lane L + E + CH, laminin incubated with the purified protease and the metalloprotease inhibitor 1,10-phenanthroline (called Component H, CH). Lane L + E + E-64, laminin incubated with the purified protease and the cysteine protease inhibitor E-64. As described in Materials and Methods, the enzyme (0.5 μg of protein, ≅ 80 kDa) was preincubated at 37° for 5 min with the inhibitor (100 μM) prior to incubation with the substrate laminin (0.5 μg, ≅ 160 kDa) at 37°. Thus the enzyme to laminin molar ratio was about 2:1. All samples were incubated for 1 hr at 37°.

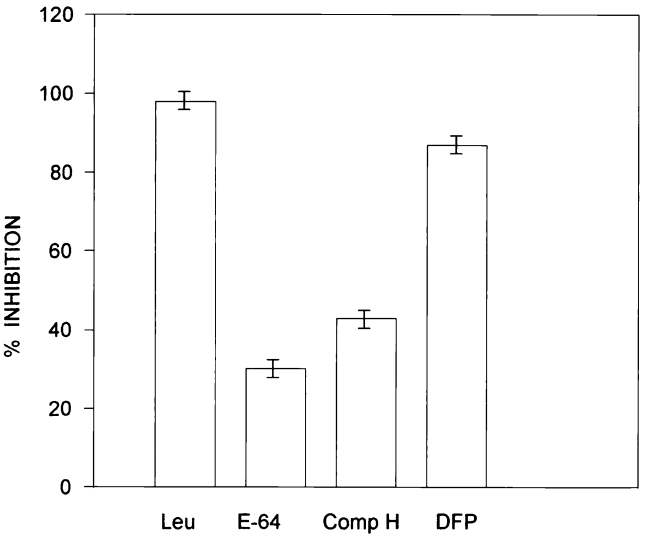

FIG. 4. Effects of different protease inhibitors on the purified protease activity, using Chromozym-TRY as a substrate (1.25 mM). The effects of the inhibitors were assayed by incubating the purified protease with the inhibitors (100 μM) at 37° for 5 min prior to enzyme assay, as described in Materials and Methods. Control protease activity was 9426.8 BAEE units/mg of protein/hr. Results are the means ± SEM of three separate experiments.

the total enzyme activity was found to be associated with the cell membrane in the absence of Triton X-100. The membrane-bound nature of the protease may have some relevance in the degradation of proteins at the dermal–epidermal junction of skin in mustard-induced pathogenesis.

Inhibition of the purified protease by the serine protease inhibitors leupeptin and DFP and the high specificity of the protease to hydrolyze the serine-specific Chromozym-TRY substrate clearly indicate that this protease contains serine at its active site.

Amino acid sequencing data suggested that the purified enzyme is a serine protease whose sequence matches the protease I precursor [14]. This protease has been shown to preferentially cleave K-X, including K-P. Although protease I was cloned and sequenced from *Achromobacter* [14], this enzyme is a mammalian-type serine protease, which bears the catalytic triad comprising His-57, Asp-113, and Ser-194 and the novel disulfide bond between Cys-6 and Cys-216. In this context, it may be mentioned that the sequences of some proteins are conserved from ciliates to humans [15]. These findings support the possibility that we have truly isolated an SM-induced serine protease from NHEK. The importance of the purified protease is indicated by its proposed involvement in the degeneration of basement membrane (with special reference to laminin) following SM exposure. This is in accord with recent observations [16–19] of laminin degradation by several proteases in certain pathological conditions. The capacity of some specific protease inhibitors to selectively prevent the proteolysis of a physiological substrate like laminin may be useful for the development of novel therapeutic approaches for the management of inflammation and blister due to SM. This neutral protease in NHEK may have an important function in the turnover of proteins present in the dermal–epidermal junction under normal conditions, and its possible potentiation, e.g. following SM exposure, may be responsible for a pathologic condition.

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