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# Chitinase Activity in Human Serum and Leukocytes

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**Using colloidal [$^3$H]chitin as a substrate, we provide the first demonstration of a chitinase in human leukocytes; chitinolytic activity in whole and disrupted leukocyte preparations (approximately 0.6 and 5.5 nmol of *N*-acetylglucosamine [GlcNAc] released min$^{-1}$ mg of protein$^{-1}$, respectively) was partially inhibited by the specific chitinase inhibitor allosamidin (9 μM). Following fractionation of the leukocytes, much higher levels of chitinase activity were detected in granulocyte-rich homogenates (approximately 7.2 nmol of GlcNAc released min$^{-1}$ mg of protein$^{-1}$) than in lymphocyte- and monocyte-rich homogenates (approximately 0.22 and 0.26 nmol of GlcNAc released min$^{-1}$ mg of protein$^{-1}$, respectively). Low levels of chitinase activity were detected in human serum (approximately 4 pmol of GlcNAc released min$^{-1}$ mg of protein$^{-1}$). Chitinolytic activity in granulocyte-rich homogenates and serum was partially inhibited by allosamidin (9 μM). Proteins with chitinolytic activities (approximate molecular masses, 48 and 56 kDa) distinct from lysozyme (14.3 kDa) were detected on polyacrylamide gels following the electrophoresis of human granulocyte-rich preparations. Chitinase activity, detected consistently in serum and leukocytes from all human volunteers investigated, may contribute to the protection of the host by cleaving chitin in the cell walls of fungal pathogens.**

Lysozyme is located in most human body fluids, including serum. As a result of its capacity to lyse peptidoglycan in the walls of bacterial pathogens, lysozyme is thought to have an important role in host defense (9). Chitin is a major structural component of the cell walls of fungal pathogens of mammals, and chitinases cleave this polymer (5). Chitinases expressed in human tissues may confer protection against fungi in a manner analogous to the protection provided by lysozyme against bacteria. Here, we report (i) the detection of a chitinase in human serum and leukocytes that cleaves colloidal [$^3$H]chitin; (ii) partial inhibition of this activity by allosamidin, a specific chitinase inhibitor (10, 18); and (iii) the detection of chitinolytic activity, distinct from lysozyme, on polyacrylamide gels following the electrophoresis of human granulocyte-rich preparations.

## MATERIALS AND METHODS

**Materials.** Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co., Poole, Dorset, United Kingdom. Allosamidin was kindly provided by Lilly Research Laboratories, Indianapolis, Ind.

**Preparation of leukocytes.** Blood was obtained from normal, healthy volunteers (five male and five female; mean age, 30 ± 2.63 years [± 95% confidence limits]) by venipuncture and mixed with preservative-free heparin (approximately 100 U of Monoparin heparin sodium [CP Pharmaceuticals] per 25 ml of blood). Erythrocytes were sedimented for 45 min at room temperature by mixing blood (10 ml) with 5 ml of 2% (wt/vol) methylcellulose–32.8% (wt/vol) sodium metrizoate (Nycomed Pharma AS, Oslo, Norway) (8:5, vol/vol). The leukocyte-rich upper fraction was aspirated and centrifuged (500 × *g* for 10 min at 4°C) to pellet the cells, which were resuspended in physiological saline (approximately 1 ml).

Lymphocytes and granulocytes were isolated from heparinized blood on Lymphoprep or Polymorphoprep gradients (Nycomed Pharma AS) according to the manufacturer's instructions.

Monocytes were obtained from whole human blood (15 ml) containing EDTA-$Na_2$ (1.5 mg ml$^{-1}$). Erythrocytes were sedimented in the presence of 1.5 ml of 6% (wt/vol) dextran 500 in physiological saline for 30 min at room temperature. Monocytes were isolated from the supernatant with Nycoprep 1.068 (Nycomed Pharma AS) according to the manufacturer's instructions.

Erythrocytes in the leukocyte fractions were removed by flash lysis following the addition of distilled water (1.8 ml) to each resuspended cell pellet, gentle mixing, and the addition of 0.2 ml of 9% (wt/vol) NaCl to restore isotonic conditions. Occasionally, it was necessary to repeat flash lysis several times to reduce the number of erythrocytes present to a negligible level.

All leukocyte suspensions were washed at least three times in physiological saline before being resuspended in physiological saline at a concentration of at least $10^6$ cells ml$^{-1}$. Cells were counted with an Improved Neubauer counting chamber.

Cell suspensions were homogenized on ice for 20 s with a 150-W ultrasonic disintegrator (MSE Ltd.) (12 μm peak to peak) and an exponentially tapered probe (3-mm tip diameter); approximately 99% of each cell population was disrupted by this procedure.

**Preparation of serum.** Serum was obtained by allowing whole human blood to clot and then centrifuging it at 500 × *g* for 10 min at 4°C; the supernatant was removed and stored at 4°C.

**Chitinase assay.** All assays were performed within 36 h of the removal of blood from a volunteer. Chitinase was assayed by the method of Dickinson et al. (4) with the following modifications. Briefly, 60 μl of tritiated chitin suspension (2.6 mg ml$^{-1}$, 1.9 μCi mg of chitin$^{-1}$, 70 kBq mg of chitin$^{-1}$) was mixed with 60 μl of enzyme preparation and either 60 μl of chitinase assay buffer (0.3 M NaCl and 50 mM Bis-Tris-HCl [pH 6.5]) for control incubations or 60 μl of chitinase assay buffer containing allosamidin (final assay concentration, 9 μM). The maximal published 50% inhibitory concentration for the inhibition of chitinase by allosamidin is 6.4 μM (16). We used a concentration of 9 μM allosamidin, as we considered this concentration to be likely to cause effective inhibition of any chitinase activity present in human blood. Incubations were for 90 min at 37°C in a shaking water bath (160 rpm), and the reaction was stopped by adding 180 μl of sodium dodecyl sulfate (SDS) (10%, wt/vol). All assays were performed in triplicate, and the variation among the replicates was never greater than 15%.

**Lysozyme assay.** Muramidase was assayed by a method based on that of Lundblad et al. (11). *Micrococcus luteus* (Sigma Chemicals) was ground with a pestle and mortar, and 100 mg was suspended in 40 ml of lysozyme assay buffer (0.1 M citrate–0.2 M phosphate buffer [pH 6.0]). After being autoclaved, this suspension was mixed with 160 ml of 1% (wt/vol) cooled agarose solution in lysozyme assay buffer, poured into petri dishes (approximately 25 ml per dish), and allowed to set, and the wells (diameter, 5 mm; six wells per plate) were bored. Hen egg white lysozyme diluted in lysozyme assay buffer was used as a positive control and to construct a standard curve. Each well contained a maximum of 70 μl of serum or cell suspension (at least $10^6$ cells ml$^{-1}$ or the equivalent) in physiological saline and allosamidin (0 or 23 μM in 50 mM Bis-Tris-HCl, pH 6.5). Plates were incubated at room temperature for 24 h, and the diameter of any zone of digestion of *M. luteus* around each well was measured.

**Protein estimation.** The protein concentration was determined by the method of Bradford (1), with bovine serum albumin being used as the standard.

**Polyacrylamide gels incorporating glycol chitin.** Glycol chitin was synthesized as described by Trudel and Asselin (20) and incorporated into polyacrylamide gels according to the method of St. Leger et al. (19). The remainder of the procedure was as described by St. Leger et al. (19), with the following modifications. Separating gels (12% polyacrylamide) and stacking gels (4.5% polyacrylamide) containing SDS (0.5%, wt/vol) were used. Samples (approximately 20 μg of protein) were diluted 1:1 (vol/vol) with nonreducing boiling mix (10% [vol/vol] stacking gel buffer [SDS (0.5%, wt/vol), 0.5 M Tris-HCl (pH 6.8)], SDS [2%,

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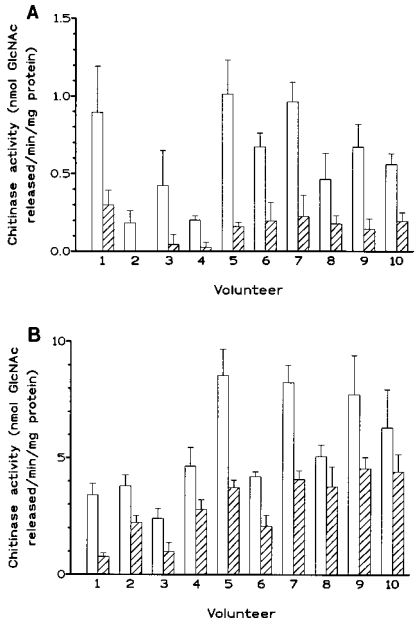


FIG. 1. Effect of allosamidin on chitinolytic activity in whole (A) and disrupted (B) leukocyte preparations from the same 10 volunteers. Leukocytes ($10^6$ cells $ml^{-1}$ or the disrupted equivalent) were assayed for chitinase activity in the presence (hatched bars) or absence (open bars) of allosamidin (9 μM) as described in Materials and Methods. Values are expressed as means ± 95% confidence limits.

wt/vol], glycerol [10%, vol/vol], bromophenol blue [0.005%, wt/vol]) and boiled for 2 min. The gel was calibrated with the following molecular markers: β-galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), triosephosphate isomerase (26.6 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa) (Boehringer Mannheim). Following SDS-polyacrylamide gel electrophoresis, the gels were shaken gently in four changes of casein buffer (0.04 M Tris-HCl [pH 9.0], 2 mM EDTA-$Na_2$, casein [1%, wt/vol], sodium azide [0.02%, wt/vol]) for 2 h at 37°C to renature the proteins (14). The gels were rinsed twice in 100 mM sodium acetate buffer, pH 4.8, and incubated in the same buffer for 15 h at 30°C. The gels were stained with Fluorescent Brightener 28 and illuminated with a UV light box. Lytic zones in which chitin had been digested appeared dark on a fluorescent white background. The gels were photographed with a GDS 2000 gel documentation system (UVP Ltd.). On completion of the procedure described above, the gels were stained for protein by fixing and staining with silver, as described by Hitchcock et al. (7).

## RESULTS

**Chitinolytic activity in human leukocytes and serum and the effect of allosamidin on enzyme activity.** With colloidal [$^3$H] chitin as the substrate, chitinolytic activity in whole-leukocyte suspensions was 0.60 ± 0.23 nmol of GlcNAc released $min^{-1}$ mg of $protein^{-1}$ (mean ± 95% confidence limit for 10 volunteers). Allosamidin (9 μM) markedly inhibited enzyme activity in the leukocyte suspensions from all 10 volunteers (Fig. 1A). Following homogenization of the leukocytes, the mean specific chitinolytic activity increased by approximately 1 order of magnitude to 5.46 ± 1.64 nmol of GlcNAc released $min^{-1}$ mg of $protein^{-1}$. Allosamidin (9 μM) inhibited chitinolytic activity in the leukocyte homogenate, although the degree of inhibition was generally not as great as that in whole-leukocyte preparations (Fig. 1A and B). The level of chitinolytic activity detected in human serum, 4.05 ± 1.48 pmol of GlcNAc released $min^{-1}$ mg of $protein^{-1}$ (mean ± 95% confidence limit for 10 volunteers), was much lower than the level of activity detected in leukocyte suspensions or homogenates; chitinolytic activity in human serum was completely eliminated after it was boiled for

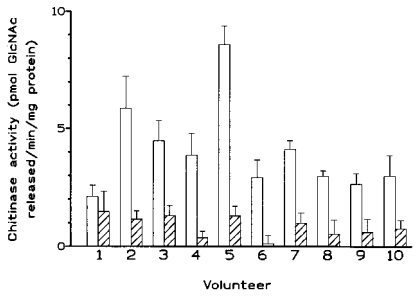


FIG. 2. Effect of allosamidin on chitinolytic activity in serum from 10 volunteers. Serum (60 μl) was assayed for chitinase activity in the presence (hatched bars) or absence (open bars) of allosamidin (9 μM) as described in Materials and Methods. Values are expressed as means ± 95% confidence limits.

2 min or incubated with SDS (10%, wt/vol). In general, allosamidin (9 μM) strongly inhibited enzyme activity in serum (Fig. 2).

When separate cell suspensions enriched for granulocytes, lymphocytes, and monocytes were homogenized, the highest specific chitinolytic activity was detected in granulocyte-rich homogenates (7.2 ± 2.87 nmol of GlcNAc released $min^{-1}$ mg of $protein^{-1}$; mean ± 95% confidence limit for three volunteers). Allosamidin (9 μM) slightly inhibited enzyme activity in granulocyte-rich homogenates from all three volunteers (Fig. 3). Much lower levels of chitinolytic activity were detected in lymphocyte- and monocyte-rich homogenates (0.22 ± 0.13 and 0.26 ± 0.14 nmol of GlcNAc released $min^{-1}$ mg of $protein^{-1}$, respectively; mean ± 95% confidence limit for three volunteers). The inhibitory effect of allosamidin (9 μM) on enzyme activity in these homogenates was, in general, not statistically significant (data not shown).

**Lysozyme activity in human leukocytes and serum and the effect of allosamidin on enzyme activity.** Maximal lysozyme activity was detected in a homogenized preparation of unfractionated leukocytes (equivalent to 29 U of hen egg white lysozyme activity mg of $protein^{-1}$ [21]). In addition, lysozyme activity was detected in serum and in all cell populations investigated during the present study (intact and homogenized lymphocytes, monocytes, and granulocytes). In keeping with the results of Koga et al. (10), allosamidin (23 μM) had no effect on lysozyme activity in serum or in any of the cell preparations investigated.

**Detection of chitinase activity on polyacrylamide gels.** When homogenized granulocyte-, lymphocyte-, and monocyte-rich preparations from three volunteers were electrophoresed on a

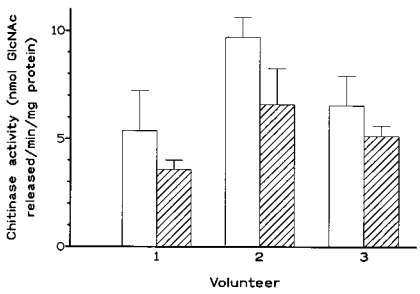


FIG. 3. Effect of allosamidin on chitinolytic activity in disrupted granulocyte preparations from three volunteers. Disrupted granulocytes (equivalent to $10^6$ cells $ml^{-1}$) were assayed for chitinase activity in the presence (hatched bars) or absence (open bars) of allosamidin (9 μM) as described in Materials and Methods. Values are expressed as means ± 95% confidence limits.

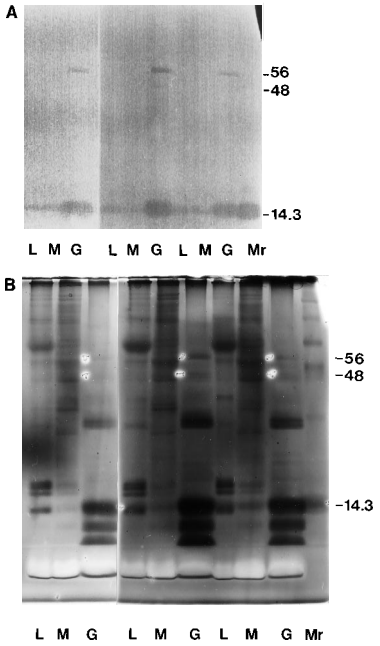


FIG. 4. (A) Chitinolytic activity detected on a 12% polyacrylamide gel containing glycol chitin. Disrupted lymphocyte (L), monocyte (M), and granulocyte (G) preparations (20 μg of protein per track) from the same three human volunteers used to generate the data shown in Fig. 3 (from left to right: volunteer 1, tracks 1 to 3; volunteer 2, tracks 4 to 6; volunteer 3, tracks 7 to 9) were electrophoresed, and chitinolytic activity (dark-staining bands) was detected as described in Materials and Methods. Track 10 (Mr) contained protein molecular mass markers, including lysozyme. The molecular masses of proteins with chitinolytic activity are indicated in kilodaltons on the right. When cell preparations were heated to 100°C for 2 min prior to dilution in boiling mix, boiling for 2 min, and electrophoresis, no chitinolytic activity was detected in glycol chitin gels. (B) The same gel shown in Fig. 4A was stained with silver. The molecular masses of proteins with chitinolytic activity detected on glycol chitin gels (Fig. 4A) are indicated in kilodaltons on the right. The light areas correspond to pinholes used to mark the $R_f$s of zones of chitinolysis detected by UV radiation.

12% polyacrylamide gel containing glycol chitin (an artificial, water-soluble substrate for chitinase in which C-6 of acetylglucosamine residues is joined in an ether linkage with a hydroxyethyl group) and the zones of chitin digestion were detected as described in Materials and Methods, a zone of lysis with an $R_f$ of 0.85 was readily apparent in each track (Fig. 4A). The highest level of lytic activity with this $R_f$ value was detected in homogenized granulocyte-rich preparations, and when the gel was calibrated with molecular weight markers, the zone of lysis was found to correspond to a protein with a molecular mass of 14.3 kDa, the molecular mass of lysozyme. Furthermore, the zone of lysis with an $R_f$ of 0.85 corresponded precisely to a zone of lysis attributable to the hen egg white lysozyme which was included in the molecular weight marker preparation (Fig. 4A).

A second major protein with chitinolytic activity with a molecular mass of approximately 56 kDa was detected following electrophoresis of granulocyte-rich but not lymphocyte- or monocyte-rich preparations on glycol chitin-containing gels (Fig. 4A). A third, apparently minor protein with chitinolytic activity with a molecular mass of approximately 48 kDa was also detected in granulocyte-rich homogenates. Although the zone of chitin digestion attributable to this protein was readily visible to the naked eye, the zone of lysis was not always apparent after the gel had been photographed with a gel documentation system (Fig. 4A). When the same gels were stained for protein, proteins with molecular masses of 48 and 56 kDa were detected in granulocyte-rich preparations from all three individuals (Fig. 4B). However, these proteins appeared to be absent from the lymphocyte- and monocyte-rich preparations (Fig. 4B).

## DISCUSSION

Using a sensitive radiometric assay with colloidal [$^3$H]chitin as the substrate, we detected an allosamidin-inhibitable chitinolytic activity in human serum and leukocytes during the present study. Colloidal chitin is a pure, unsubstituted, and specific substrate for chitinase, while allosamidin is a highly specific inhibitor of chitinase activity (10). Therefore, our results represent the first clear demonstration of a chitinase in human blood cells. Most of the chitinolytic activity in human blood appeared to be present in granulocytes; the amount of enzyme activity detected by the radiometric assay appeared to be directly proportional to the level of chitinolytic activity exhibited by enzymes with apparent molecular masses of 48 and 56 kDa (Fig. 3 and 4). Little is known of the cellular distribution of chitinolytic enzymes in the blood of other mammals. However, Manson et al. (13) detected chitinase activity in turbot leukocytes; they also detected chitinase in turbot erythrocytes at levels approximately 2 orders of magnitude less than those detected in the leukocytes. Chitinase activity has been detected in sera from a range of animals; the molecular masses of bovine and goat serum chitinases were estimated as 47 and 60 kDa, respectively (11, 12).

Hakala et al. (6) characterized human cartilage glycoprotein 39 (gp39), a major secretory product of articular chondrocytes and synovial cells. Comparison of the deduced amino acid sequence of this protein with known sequences revealed that human cartilage gp39 contains regions displaying significant homology with sequences of chitinases from a range of organisms. However, human cartilage gp39 did not display any glycosidase activity against a number of chitinase substrates, indicating that the glycoprotein (molecular mass, 39 kDa) is distinct from the chitinase(s) detected during the present investigation.

Human plasma contains both a chitotriosidase that is distinct from lysozyme (8) and a chitotetraosidase that appears to be distinct from lysozyme, hyaluronidase, neutral endoglucosaminidase, *N*-acetyl-β-D-hexosaminidase, aspartylglucosaminidase, β-D-glucosidase, and chitobiase activities (2, 3). Neither Den Tandt et al. (2, 3) nor Hollak et al. (8) determined the molecular weight of human serum chitotetraosidase or chitotriosidase, respectively. Hollak et al. (8) raised the possibility that the chitotriosidase activity they detected in human serum and the chitotetraosidase characterized by Den Tandt et al. (2, 3) may be attributable to the same enzyme. Most recently, Renkema et al. (17) purified two chitotriosidases (molecular masses, 39 and 50 kDa) from the spleen of a patient suffering from Gaucher disease. The N-terminal amino acid sequences were identical; both the N-terminal sequence and an internal sequence of the 39-kDa protein proved to be homologous to those of proteins that are members of the chitinase family. Furthermore, the chitotriosidases demonstrated activity against chitin azure substrate. The results of another recent study provide further evidence for the presence of chitinase in humans. Overdijk and Van Steijn (15) found that allosamidin inhibited chitotriosidase detected in serum and demonstrated that the enzyme had activity against chitin and ethylene glycol chitin substrates. Other workers have shown that chitinases cleave both chitotriosidase and chitotetraosidase substrates (5), and it seems likely that the chitinolytic activities detected during the present study are closely related to the chitotriosi-

dase and chitotetraosidase activities detected by Den Tandt et al. (2, 3) and Hollak et al. (8) and the chitotriosidase and chitinase activities detected by Overdijk and Van Steijn (15) and Renkema et al. (17).

The molecular masses of the human spleen enzymes detected by Renkema et al. (39 and 50 kDa) (17) differ from the molecular masses of the human granulocyte enzymes (48 and 56 kDa) detected during the present study. However, it should be emphasized that granulocyte chitinases were detected on a chitin-containing polyacrylamide gel with proteins prepared under nonreducing conditions. In contrast, Renkema et al. (17) determined the molecular weights of the spleen enzymes on gels which did not contain chitin, and these authors apparently used proteins prepared under reducing conditions. Furthermore, Renkema et al. (17) investigated chitotriosidase and chitinase activities in the spleen of only one individual; we detected chitinase activity in granulocytes from six volunteers and noted some slight variation in the molecular masses of these enzymes among individuals (data not shown). Hollak et al. (8) found that some individuals lacked chitotriosidase activity. However, the fluorimetric assay they used to assay chitotriosidase activity is likely to have been much less sensitive than the specific radiometric assay for chitinase used during the present study; this may explain why we detected chitinase activity in all of the human volunteers investigated.

Chitin is a major structural component of the cell walls of important fungal pathogens of man. Neutrophils are granulocytes that perform an important defensive role by ingesting and killing microbial pathogens. Thus, the high levels of chitinase detected consistently in human granulocytes during the present study may reflect the fact that the enzyme makes an important contribution to the destruction of pathogens in neutrophils by cleaving the chitin of the fungal cell wall.

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