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Purification and Characterization of Human Chitotriosidase, a Novel Member of the Chitinase Family of Proteins*

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Recently we noted (Hollak, C. E. M., van Weely, S., van Oers, M. H. J., and Aerts, J. M. F. G. (1994) *J. Clin. Invest.* 93, 1288–1292) that the clinical manifestation of Gaucher disease is associated with a several hundred-fold increase in chitotriosidase activity in plasma. We report on the purification and characterization of the protein.

Two major isoforms of chitotriosidase with isoelectric points of 7.2 and 8.0 and molecular masses of 50 and 39 kDa, respectively, were purified from the spleen of a Gaucher patient. The N-terminal amino acid sequence of the two forms proved to be identical. An antiserum raised against the purified 39-kDa chitotriosidase precipitated all isozymes. Chitotriosidase activity was earlier found to be completely absent in some individuals. These findings in combination suggest that a single gene may encode the different isoforms of chitotriosidase.

Both the N-terminal sequence and an internal sequence chitotriosidase proved to be homologous to sequences in proteins that are members of the chitinase family (Hakala, B. E., White, C., and Recklies, A. D. (1993) *J. Biol. Chem.* 268, 25803–25810). The human chitotriosidase described here showed chitinolytic activity toward artificial substrates as well as chitin and may therefore be considered to be a chitinase.

Gaucher disease is a recessively inherited lysosomal storage disorder in which the activity of the enzyme glucocerebrosidase is markedly decreased. This results in accumulation of the glycolipid glucosylceramide in the lysosomes of macrophages (1). Recently, enzyme-replacement therapy has been successfully applied by infusing purified placental glucocerebrosidase, which has been modified so as to target the enzyme to macrophages (2). The clinical manifestation of Gaucher disease is generally accompanied by increased plasma levels of certain enzyme activities, including acid phosphatase 5B (3), angiotensin-converting enzyme (4, 5), lysosomal hydrolases (6, 7), and lysozyme (5, 8). For instance, there is an approximately 10-fold increase in the activity of acid phosphatase 5B in plasma of Gaucher patients compared with that of controls (see *e.g.* Ref. 9); the elevations in the activity of other enzymes are much less pronounced.

Chitotriosidase activity was found to be on average more than 600 times increased in plasma of Gaucher patients compared with controls (9). Such a marked elevation has, so far, been observed only in samples from Gaucher patients and not in plasma from patients with other pathological conditions. Moreover, successful therapeutic intervention in Gaucher disease proved to be accompanied by a rapid and marked reduction in the chitotriosidase levels in plasma (9). In our previous study it was observed that chitotriosidase is a secretory protein of cultured macrophages (9). A small amount of enzyme is also found intracellularly, possibly in lysosomes. The enzyme does not show the characteristic acid pH optimum of lysosomal enzymes but has very similar activity in the pH range 3–8.

Human chitotriosidase had not been purified so far, and little is known about the nature and function of the enzyme. Furthermore, the relationship between the several hundred-fold increased plasma levels of chitotriosidase and the pathophysiology of Gaucher disease is unclear. Here, we report on the purification of chitotriosidase from Gaucher spleen and describe a number of characteristics of the enzyme.

MATERIALS AND METHODS

Enzyme Assays—Chitotriosidase and chitobiosidase activities were routinely determined with the fluorogenic substrates 4MU[1]-chitobiose (Sigma, 4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobiose) and 4MU-chitotriose (Sigma, 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotriose). Substrate mixtures contained 0.027 mM substrate and 1 mg/ml bovine serum albumin in McIlvain buffer (100 mM citric acid, 200 mM sodium phosphate), pH 5.2. Assays were performed as described previously (9).

The two PNP substrates (Sigma, *p*-nitrophenyl β-D-*N*,*N*′-diacetylchitobiose and *p*-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose) were used in McIlvain buffer (pH 5.2) at a concentration of 370 and 270 μM, respectively. Assays (final volume, 100 μl) were stopped with 50 μl of 3 M glycine-NaOH buffer (pH 10.6). The *p*-nitrophenyl formed was determined spectrophotometrically at 405 nm.

Chitinase activity was determined using chitin azure (Sigma), which was suspended in McIlvain buffer (pH 5.2). The final concentration of chitin azure particles was 10 mg/ml. Degradation was monitored by spectrophotometric detection at 550 nm of soluble azure after centrifugation (10). Chitinase from *Serratia marcescens* (Sigma) was used as a control.

Lysozyme activity was determined according to Mörsky (11) by measuring the decrease in absorbance at 450 nm of a *Micrococcus lysodeikticus* suspension (Sigma, 0.26 mg/ml) in McIlvain buffer (pH 5.2). Lysozyme from human milk (Sigma) was used as a control.

Purification of Chitotriosidase—Detergent-free spleen extract was prepared by homogenization of Gaucher type I spleen in 4 volumes of water, using an Ultra-turrax and centrifugation for 20 min at 15,000 × *g*. The pH of the supernatant was adjusted to 8.5 using 1 M Tris buffer (final concentration, 25 mM), and the supernatant was applied to a polybuffer exchange column (PBE 94, Pharmacia Biotech Inc.); the column was equilibrated and eluted with 25 mM Tris buffer (pH 8.5). Breakthrough fractions with highest chitotriosidase activity were pooled and concentrated by Amicon PM10 ultrafiltration. This pool was

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[1] The abbreviations used are: 4MU, 4-methylumbelliferyl; PNP, *p*-nitrophenyl; PBE, polybuffer exchanger; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

applied to a Sephadex G-100 (Pharmacia) column and eluted with 25 mM Tris buffer (pH 8.0). Fractions were collected and peak fractions containing enzyme activity were pooled and concentrated again. As a final step in the isolation procedure preparative isoelectric focusing was performed.

Protein concentrations were determined according to the method of Lowry *et al.* (12), using bovine serum albumin as standard.

Isoelectric Focusing—Preparative flat-bed isoelectric focusing was performed using Ultrodex (Pharmacia) containing 0.5% (v/v) Triton X-100 and 0.1% w/v ampholytes (Servalyte 4–9, Serva). Focusing was performed overnight at 10 °C at 400 V, using an LKB 2117 Multiphor apparatus as described by the manufacturer. The gel was fractionated and extracted with water, after which the chitotriosidase activity and pH of the fractions were determined.

Chitotriosidase Fragmentation by Proteolytic Digestion—Purified chitotriosidase was denatured by boiling in 1% β-mercaptoethanol and 0.5% SDS. Digestion was done with trypsin (Boehringer Mannheim) (chitotriosidase:trypsin, w/w, was about 100:1) at room temperature for 5 min and stopped by boiling in SDS-PAGE sample buffer.

SDS-PAGE—SDS-PAGE was performed on a Pharmacia Phast-gel system, according to the manufacturer's instructions, using 12.5% (w/v) acrylamide gels. After electrophoretic separation the gels were silver-stained. For the separation of proteins prior to sequencing, 10% SDS-PAGE gels were used according to the method of Laemmli (13). Protein digests were separated on 12% gels.

Protein Sequencing—Protein samples were separated on SDS-PAGE and blotted to polyvinylidene difluoride membrane (Bio-Rad) using a blotting buffer containing 50 mM Tris, 50 mM borate, 20% (v/v) methanol, and 0.02% (w/v) SDS (pH 8.1–8.5). Blots were stained with 1% (w/v) Coomassie Brilliant Blue (R-250) in 50% v/v methanol, destained with 10% acetic acid in 50% methanol, and dried. Protein bands were applied to a Beckman/Porton LF 3000 protein sequencer coupled to a Beckman System Gold phenylthiohydantoin analyzer. The sequences obtained were compared with those present in the EMBL data bank.

Determination of Native Molecular Weight by Gel Filtration—Sephadex G-100 (Pharmacia) gel filtration was used to determine the native molecular weight of chitotriosidase. A column was calibrated using the following proteins as standards: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and alcohol dehydrogenase (150 kDa).

Immunoprecipitation—Immunoprecipitation of chitotriosidase with immobilized antibodies was performed as described in Ref. 9.

RESULTS

Purification of Chitotriosidases—Chitotriosidase activity was found to be about 50-fold increased in spleen of a type I Gaucher patient as compared with spleen from a control subject. The enzyme activity was completely recovered in the supernatant of a detergent-free extract that was used as starting material for the isolation of chitotriosidase. The extract was applied on a polybuffer exchange chromatofocusing column (PBE 94), equilibrated to pH 8.5. The chitotriosidase activity was not bound and only slightly retained by the column, in contrast to total protein. The fractions enriched in enzyme activity were pooled and, after concentration via ultrafiltration, subjected to Sephadex G-100 gel filtration. Fractions containing chitotriosidase activity were pooled, concentrated, and subjected to isoelectric focusing. Measurement of chitotriosidase activity in the fractionated gel showed the presence of two distinct forms of chitotriosidase with apparent pIs of 7.2 and 8.0, respectively (not shown). Analysis by SDS-PAGE and silver staining showed that the pI 8.0 fraction contained a protein with an apparent molecular mass of 39 kDa (Fig. 1, *lane 4*). Depending on the fractionation of the gel, minor amounts of other proteins were sometimes noted in chitotriosidase-containing fractions in the pI range 7.9–8.2. However, chitotriosidase activity in such fractions always correlated with the concentration of the 39-kDa protein only. The chitotriosidase-containing fractions with pI around 7.2 contained several proteins with apparent molecular masses of 50, 42, 25, and 18 kDa (Fig. 1, *lane 5*).

The native molecular masses of the pI 8.0 and 7.2 chitotriosidases were 29 and 37 kDa, respectively, on a calibrated Sephadex G-100 column (not shown).

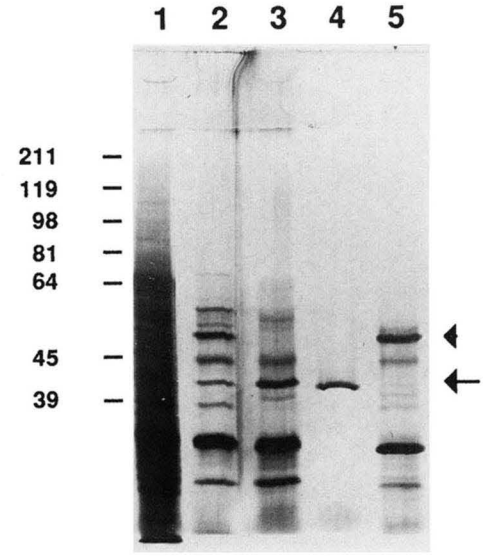

FIG. 1. **Analysis of protein constituents of fractions of a typical chitotriosidase purification procedure.** Proteins were separated on 12.5% SDS-PAGE gel and visualized by silver staining. Molecular mass standards are indicated (kDa). *Lane 1*, spleen extract; *lane 2*, pool of PBE column; *lane 3*, pool of Sephadex G-100 column; *lane 4*, IEF fraction with pH of 8.0; *lane 5*, IEF fraction with pH of 7.2. The 39-kDa isoform of chitotriosidase is indicated by an *arrow* and the 50-kDa isoform by an *arrowhead*.

Table I gives the results overview of a typical isolation. The amount of 39-kDa chitotriosidase in the final pI 8.0 fraction was determined by silver staining and comparison with known amounts of bovine serum albumin. The isolation procedure resulted in a more than 3600-fold purification of the 39-kDa (pI 8.0) chitotriosidase from an extract of a spleen from a type I Gaucher patient. Four independent isolations gave comparable results.

Amino Acid Sequences of Chitotriosidases—The sequence of the first 22 N-terminal amino acids of the 39-kDa protein with pI 8.0 is presented in Fig. 2. In the fraction containing chitotriosidase activity with pI of about 7.2, only the 50-kDa protein could be sequenced. The first 22 amino acids at the N terminus of this protein were identical to those at the N terminus of the 39-kDa (pI 8.0) protein. Digestion of the purified 39-kDa chitotriosidase with trypsin resulted in a characteristic pattern of fragments. An internal sequence of 21 amino acids was obtained from a digestion fragment of 30-kDa protein.

Homology was proven to exist between the N-terminal and internal sequences of the human chitotriosidase and those of proteins that are members of a recently recognized chitinase protein family (14), as shown in Fig. 2. This family consists of proteins from various organisms, with strong homology in several domains including the region that is involved in the catalysis of the hydrolysis of chitin and the artificial substrate 4MU-chitotrioside (15).

Chitotriosidase Isozymes—Besides the two predominant forms of chitotriosidase with pI 7.2 and 8.0, isoelectric focusing of several Gaucher spleen extracts revealed minor forms of pI 5.5–6.0 and pI about 6.5 (Fig. 3*A*). The apparent isoelectric point of chitotriosidase activity in Gaucher plasma was predominantly 7.2, with minor amounts of 6.0 and 8.0 (Fig. 3*B*). The isoelectric focusing profiles of chitotriosidase in corresponding control materials were comparable (not shown).

To study the relationship between the various chitotriosidase isozymes, an antiserum was raised in a rabbit against purified native 39-kDa enzyme. This antibody recognized only native chitotriosidase, and more than 98% of the chitotriosi-

TABLE I

Isolation of pI 8.0 chitotriosidase from spleen of a type I Gaucher patient

Data from a typical isolation are presented. Four independent isolations gave comparable results.

Fraction	Volume	Protein	Activity	Specific activity	Recovery	Purification
	ml	*mg/ml*	*nmol/ml·h^{-1}*	*nmol/mg·h^{-1}*	*%*	*-fold*
Detergent-free extract	110	5.96	10,300	1730	100	1.0
PBE chromatofocusing	77	0.21	7830	37,220	53	21.5
Sephadex G-100 gel filtration	28.5	0.24	17,030	72,155	43	42
Preparative IEF	1.8	0.02	141,180	6,274,700	22	3630

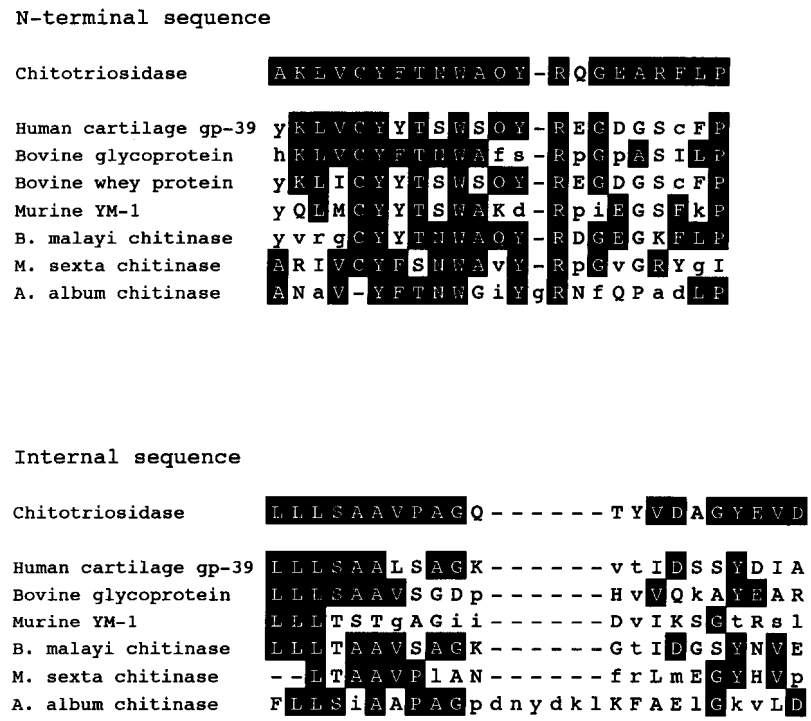

FIG. 2. **N-terminal amino acid sequence and an internal amino acid sequence of chitotriosidase; alignment with members of the chitinase protein family.** The N-terminal sequence was determined for both the 39- and 50-kDa isoforms of chitotriosidase and proved to be identical. The internal sequence was obtained from a tryptic fragment of the 39-kDa isoform of chitotriosidase. The proteins are: HC gp-39, a human glycoprotein produced by chondrocytes and synovial cells (GenBank M80927); a bovine oviduct-specific glycoprotein (GenBank D16639); a protein secreted in bovine whey during involution (SwissProt P30922; only the N-terminal amino acid sequence of this protein is available); YM-1, a secretory protein of activated mouse macrophages (Pir S27879); an endochitinase of the nematode *B. malayi* (SwissProt P29030); a chitinase of the hornworm *Manduca sexta* (GenBank U02270); and a chitinase of the fungus *A. album* (SwissProt P32470). Residues identical to chitotriosidase are indicated by *white letters*; *capital letters* indicate residues with similar properties to those in chitotriosidase.

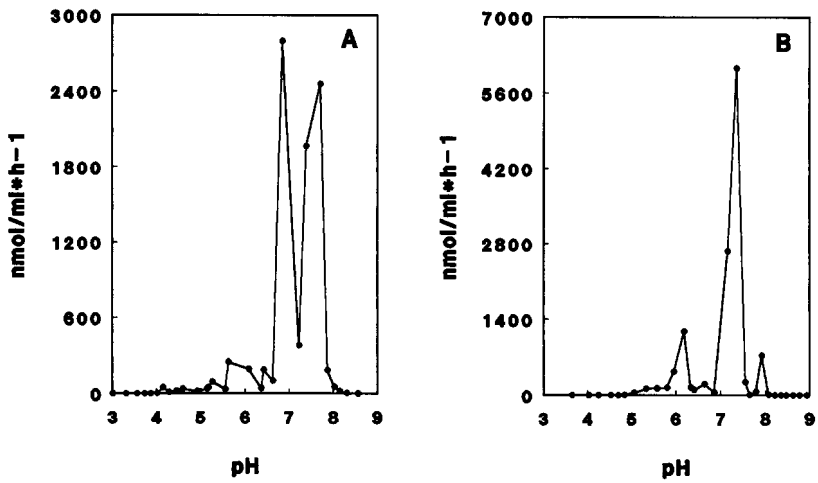

FIG. 3. **Isoelectric focusing profiles of chitotriosidase activity in Gaucher materials.** Isoelectric focusing was performed as described under "Materials and Methods." Chitotriosidase activity was measured with the 4MU-chitotrioside substrate. *A*, Gaucher spleen extract; *B*, Gaucher plasma sample.

dase activity in the Gaucher spleen extract was immunoprecipitable with this immobilized anti-(39-kDa chitotriosidase) antiserum. Chitotriosidase in pI 8.0, 7.2, and 5.5–6.0 fractions was identically precipitated in immunotitration experiments (not shown).

Earlier we found that some individuals are deficient in plasma chitotriosidase activity (9). We observed that a deficiency in plasma was accompanied by a deficiency in other materials, such as spleen. The deficiency was not due to the presence of some inhibitor but probably the result of some inherited defect. These observations suggest that the different chitotriosidase isozymes are most likely encoded by a single gene.

All lysosomal hydrolases, with the exception of lysozyme, contain *N*-linked glycans that bind strongly to either the lectin concanavalin A or the lectin *Ricinus communis* agglutinin. When tested, chitotriosidase showed no affinity for binding to these two lectins (not shown). Incubation of pure 39-kDa chitotriosidase with endoglycosidases H and F or *N*-glycanase also did not result in a change in apparent molecular mass. Fur-

TABLE II

Hydrolysis of substrates by human chitotriosidases, human lysozyme, and bacterial chitinase

Chitinase activity was determined with chitin azure as substrate; lysozyme activity was determined with the *M. lysodeikticus* cell wall suspension assay. Assays were performed as described under "Materials and Methods." The lysozyme had been purified from human milk and the chitinase from *S. marcescens*. ND, not determined.

Enzyme	4MU-chitobioside	4MU-chitotrioside	Chitinase activity	Lysozyme activity	PNP-chitobioside	PNP-chitotrioside	Bio/trio ratio 4-MU	Bio/trio ratio PNP
	$nmol/ml \cdot h^{-1}$		$A_{550}/ml \cdot h^{-1}$	*units*	$nmol/ml \cdot h^{-1}$			
pI 8.0 chitotriosidase	25,904	33,371	52.1	0	838.7	216.1	0.8	3.9
pI 7.2 chitotriosidase	13,928	20,020	45.8	ND	541.1	178.2	0.7	3.0
Human lysozyme	0	6	20.8	2140	0.7	4.7		0.1
Bacterial chitinase	123,100	118,000	306.9	2	19,532.3	21,725.8	1.0	0.9

thermore, preliminary results of metabolic labeling experiments with cultured macrophages revealed no shift in mobility upon addition to the culture medium of tunicamycin (not shown), again suggesting the absence of *N*-linked glycosylation.

Hydrolysis of Substrates—The results of experiments on the substrate specificity of chitotriosidase are shown in Table II. Purified samples of different pI forms showed a higher activity toward 4MU-chitotrioside substrate than toward 4MU-chitobioside, the ratio of chitobioside/chitotrioside activity of all chitotriosidase preparations being about 0.7. Both PNP-chitobioside and PNP-chitotrioside were hydrolyzed by purified chitotriosidase. However, in the case of the PNP-substrates the chitobioside substrate was more rapidly hydrolyzed.

Since 4MU-chitotrioside has been reported to be a substrate for lysozyme (16), the activity was studied of purified chitotriosidase toward a suspension of cell walls of *M. lysodeikticus*, a natural substrate for lysozyme. Purified chitotriosidase showed no lysozyme activity, as shown in Table II.

Because of the high degree of homology of chitotriosidase with a number of chitinases, it was of interest to study the capacity of chitotriosidase to degrade chitin, a polymer of β-1,4-linked *N*-acetylglucosamine moieties. Chitin azure was used as substrate. Table II shows that chitin azure was, indeed, a substrate for this enzyme. When related to the hydrolysis of 4MU-chitotrioside, degradation of chitin azure by the human chitotriosidase was even better than by the bacterial chitinase studied.

DISCUSSION

In this report we describe the purification and partial characterization of the newly discovered human chitotriosidase that is highly elevated in Gaucher patients (9). The chitotriosidase characterized by us may be identical to a human plasma 4-methylumbelliferyl-tetra-*N*-acetylchitotetraose hydrolase described by Den Tandt and co-workers (17, 18). These investigators found that their partially purified enzyme did not exhibit hyaluronidase, neutral endoglucosaminidase, aspartylglucosaminidase, β-hexosaminidase, β-glucosidase, or chitobiase activity. We, too, were unable to demonstrate any β-hexosaminidase or β-glucosidase activity for the purified chitotriosidase. Nor was the enzyme able to hydrolyze the β-1–4 linkage between *N*-acetylglucosamine and muramic acid in cell walls from *M. lysodeikticus*, and thus it clearly differs from lysozyme. The relatively high enzymatic activity toward chitin suggests that our human chitotriosidase may be considered to be a functional chitinase. Indeed, sequencing of the N terminus and a digestion fragment of purified human chitotriosidase revealed that this protein shares homology with chitinases from non-mammalian organisms, *e.g.* the nematode *Brugia malayi* (19) or the fungus *Aphanocladium album* (20).

We noted that the human chitotriosidase was still active at 50 °C and could be inhibited by Ag^+. Similar properties have been documented for the chitinase (Ch1) of *A. album* (see Ref. 21, and references therein).

Our finding that chitotriosidase is a chitinase is of particular importance since, even in recent publications (see *e.g.* Ref. 22), the human body is still believed to contain no chitin.

Recently it has been recognized that not only do chitinases from various non-mammalian organisms (such as bacteria, fungi, plants, and insects) share structural homology but proteins with a partially similar structure also occur in mammals. The members of the so-called chitinase protein family (14) differ in ability to catalyze the hydrolysis of chitin or chitin-like substrates such as 4-methylumbelliferyl chitotrioside. All documented mammalian members of the family have been found, so far, to be without chitinolytic activity. These mammalian proteins include a human cartilage protein (HC gp-39) (14, 23), a murine protein secreted by activated macrophages (YM-1; only documented in the Pir data bank), a bovine whey protein (24), as well as a baboon (25) and a bovine oviduct-specific glycoprotein (26). Their inability to hydrolyze substrate is most likely explained by the absence of critical acidic amino acids in the catalytic site region (15), as can be deduced from the nucleotide sequence of cDNA encoding HC gp-39, YM-1, and bovine oviduct-specific glycoprotein. The chitotriosidase isolated from Gaucher spleen clearly differed from the other mammalian members of the chitinase protein family. This protein appears to be more closely related to the chitinases of non-mammalian organisms, since it is also a functional chitinolytic enzyme.

The human chitotriosidase described here may be involved in defense against and in degradation of chitin-containing pathogens such as fungi, nematodes, and insects. The function of the members of the chitinase protein family without chitinase activity is unknown. Some, such as HC gp-39 and the bovine whey protein, are expressed in association with remodeling events (14). Interestingly, in plants, chitinases are believed to be involved in defense against pathogens as well as morphogenetic processes that involve remodeling (see Ref. 21, and references therein). The role of the chitinases in morphogenesis is poorly understood since plants do not contain endogenous chitin. It cannot be excluded that, in analogy to the situation in plants, chitotriosidase in man also fulfills multiple functions.

The relationship of the various chitotriosidase isozymes that occur in man is not precisely understood. However, the finding that all chitotriosidase activity is absent in some individuals (9) suggests that this enzyme is encoded by a single gene. This suggestion is in agreement with our present finding that an antibody raised against the 39-kDa chitotriosidase precipitated all isozymes. Moreover, the N terminus of at least the 39- and 50-kDa isozymes was identical. The heterogeneity in chitotriosidase could therefore be due to alternative splicing, post-translational proteolytic processing, or differences in glycosylation.

Further information about the structure, the regulation of synthesis, and the routing of human chitotriosidase as well as its physiological substrate is required in order to be able to

understand the role of the enzyme under normal and pathological conditions. Cloning of the corresponding cDNA and analysis of the processing of the protein are, therefore, being undertaken. These investigations will be crucial to the identification of possible effects of the relatively common deficiency in enzyme activity in man (9) and to identify the cause and consequences of the strong increase in plasma levels of chitotriosidase in clinically affected Gaucher patients.

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