ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 229, No. 1, February 15, pp. 43-54, 1984

Oligosaccharides on Cathepsin D from Porcine Spleen[1]

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Received June 6, 1983, and in revised form September 8, 1983

Cathepsin D from porcine spleen contained mannose (3.3%), glucosamine (1.4%), and mannose 6-phosphate (0.08%). Essentially all of the oligosaccharides of cathepsin D could be released by endo-β-*N*-acetylglucosaminidase H, pointing to oligomannoside types of structures. Three neutral oligosaccharide fractions, containing 5, 6, and 7 mannose residues, respectively, were isolated by gel permeation chromatography on Bio-Gel P-2. Studies using exoglycosidase digestions and 500-MHz ^{1}H NMR spectroscopy revealed that their structures are [Manα1 $\rightarrow$ 2]$_{0\text{ or }1}$Manα1 $\rightarrow$ 6[Manα1 $\rightarrow$ 3]Manα1 $\rightarrow$ 6[(Manα1 $\rightarrow$ 2)$_{0\text{ or }1}$Manα1 $\rightarrow$ 3]Manβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 4 GlcNAc. These structures are identical to what have recently been proposed by Takahashi *et al.* for the major oligosaccharide units of cathepsin D from the same source (T. Takahashi, P. G. Schimidt, and J. Tang (1983) *J. Biol. Chem.* **258**, 2819-2930), except for the occurrence of two isomeric oligosaccharides containing six mannoses. Only a part (3.4%) of the oligosaccharides were acidic, containing phosphates in monoester linkage. The phosphorylated oligosaccharides also consisted of oligomannoside-type chains which were analogous to, but more heterogeneous in size than the neutral oligosaccharides. Cathepsin D was bound to a mannose- and *N*-acetylglucosamine-specific lectin (mannan-binding protein) isolated from rabbit liver with the K_i value of 5.4×10^{-6} M.

Carbohydrate recognition systems which mediate transport of lysosomal enzymes have been studied intensively during many years. In fibroblasts, transport of lysosomal enzymes to the lysosomal particles from the site of biosynthesis and extracellular milieu has been shown to be mediated by a system recognizing the mannose 6-phosphate moiety in oligomannoside-type oligosaccharides (1-5). In alveolar macrophages and sinusoidal cells of the liver, endocytosis of lysosomal enzymes is mediated by a system specific for mannose, fucose, and *N*-acetylglucosamine (6-11). Parenchymal cells of the liver contain a mannose- and *N*-acetylglucosamine-specific binding protein which binds lysosomal enzymes with high affinity (11-16).

In contrast to the extensive characterization of these receptor systems, information about the structure of oligosaccharides on lysosomal enzymes is very limited. Therefore, we studied the oligosaccharide structure of cathepsin D, a major lysosomal enzyme. Cathepsin D from porcine spleen (M_r = 50,000) consists of a light (M_r = 15,000) and a heavy (M_r

[1] This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan; by the Mitsubishi Foundation; by the Netherlands Foundation for Chemical Research (SON/ZWO); and by Grant UUKC-OC 79-13 from the Netherlands Foundation for Cancer Research (KWF).

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0003-9861/84 $3.00

= 35,000) chain, both of which are glycosylated (17). The carbohydrate portion of cathepsin D seems to be involved in the correct delivery of the enzyme into the lysosomal compartment and in the proper proteolytic processing from procathepsin D to the mature enzyme in cultured rat liver cells (18) and porcine kidney cells (19). During the course of preparation of this manuscript, a report of Takahashi *et al.* (20) has appeared, in which they showed a single glycosylation site in L- and H-chains of cathepsin D from porcine spleen, and elucidated their oligosaccharide structures by ^{1}H NMR analysis of glycopeptides from chymotryptic and thermolytic digestion. In the present work, cathepsin D was digested with Pronase and the digests were treated with endo-β-*N*-acetylglucosaminidase H. The released oligosaccharides were purified by high-voltage paper electrophoresis and gel permeation chromatography and analyzed with exoglycosidase digestions and ^{1}H NMR spectroscopy. The results confirm and establish the structures of three major oligosaccharides of oligomannoside type Takahashi *et al.* (20) proposed, with quantitative data obtained by high-resolution ^{1}H NMR spectroscopy on the extensively purified oligosaccharides. In addition, we extended their studies by revealing the occurrence of the isomeric oligosaccharides containing six mannoses and of phosphorylated oligosaccharides. A preliminary account of this has been published (21) and the details are now presented.

MATERIALS AND METHODS

All the information in this section, plus Figs. 1–5, 7, and 8, and Tables I and II, are presented in the miniprint supplement which accompanies and is part of this manuscript.

RESULTS

Carbohydrate composition of cathepsin D. As shown in Table I, carbohydrate of cathepsin D from porcine spleen was composed almost entirely of mannose and glucosamine, suggesting that the enzyme consists of oligomannoside-type oligosaccharides. The results were in agreement with the observations of Huang *et al.* (17). A small quantity of mannose 6-phosphate, 0.08%, detectable on the enzyme indicated that one-sixth of the cathepsin D molecules could contain one phosphorylated sugar.

Isolation of oligomannoside-type oligosaccharides. Exhaustive digestion of cathepsin D with Pronase gave rise to a single glycopeptide peak on a Sephadex G-25 column (1.4 × 115 cm) as shown in Fig. 2A. Upon digestion of the glycopeptides with endo-β-*N*-acetylglucosaminidase H, the hexose peak shifted to a lower-molecular-weight range (B compared to A in Fig. 2), being consistent with 80% release of oligosaccharides as estimated by the increment of reducing activity, whereas approximately 5% of the total hexose was recovered in the high-molecular-weight margin (tube numbers 50–58). Upon high-voltage paper electrophoresis, the oligosaccharides were separated into neutral (fraction 1, 96%) and acidic (fraction 2, 4%) components (Fig. 3), with a total recovery of approximately 80%. In this step, the neutral oligosaccharides were almost completely separated from contaminating peptides which might disturb the following analyses including NMR studies.

Separation and characterization of neutral oligosaccharides. Gel permeation chromatography of the neutral oligosaccharides (fraction 1, Fig. 3) on a Bio-Gel P-2 column resulted in three subfractions in the ratio of 22, 30, and 48% for N-1, N-2, and N-3, respectively (Fig. 4A). The most predominant peak (N-3) was eluted at the position of $Man_5GlcNAcol$, and N-2 and N-1 earlier than $Man_5GlcNAcol$ by one and two additional hexose units, respectively (22). Also, by paper chromatography using Solvent I, the neutral oligosaccharides could be resolved into three major subfractions, the position of the fastest moving peak being identical with that of $Man_5GlcNAcol$ (Fig. 5). Neutral oligosaccharides were further characterized by various exoglycosidase digestions. Treatment with *Aspergillus saitoi* $\alpha 1 \rightarrow 2$-specific mannosidase of the mixture of the neutral oligosaccharides (N-1 + N-2 + N-3) gave a single peak at the elution position of N-

3 (Fig. 4B), revealing that N-1 and N-2 contained one and two additional $\alpha 1 \rightarrow 2$-linked mannose residues at the nonreducing termini of N-3, respectively. Digestion of the products with jack bean α-mannosidase produced a single component eluted at the position of ManGlcNAcol, revealing that four α-mannose residues were attached to the ManGlcNAcol core (Fig. 4C). Most of the jack bean α-mannosidase digests were, in turn, converted into GlcNAcol by digestion with β-mannosidase (Fig. 4D). Based on these lines of evidence and taking the specificity of endo-β-*N*-acetylglucosaminidase H (23–25) into account, the structure of the neutral oligosaccharides can be presented as $[\text{Man}\alpha 1 \rightarrow 2]_n\alpha\text{Man}_4\beta\text{ManGlcNAc}\beta 1 \rightarrow$ 4-GlcNAc (n = 2, 1, and 0 for N-1, N-2, and N-3, respectively).

Further characterization of each neutral oligosaccharide was carried out using 500-MHz ^{1}H NMR spectroscopy. The pertinent structural-reporter group regions (26), namely, the H-1 and the H-2 resonances, of the resolution-enhanced 500-MHz ^{1}H NMR spectra of the three oligosaccharide fractions are presented in Fig. 6. Relevant ^{1}H chemical shifts for the three samples are listed in Table II.

Comparison of the spectral data for N-1, N-2, and N-3 (Table II) reveals that the resonance positions for H-2, and also for the *N*-acetyl methyl protons, of GlcNAcol (designated 2ol) in each spectrum are identical. The Man-3 residue, which is $\beta 1 \rightarrow$ 4-linked to GlcNAc-2ol, is clearly characterized by its H-2 signal at $\delta \cong 4.25$. This resonance is typically shaped, namely, as a broad-lined doublet, which indicates the β-type of linkage. The chemical shift value for this H-2 points unambiguously to a disubstitution of Man-3 at C-3 and C-6, by Man-4 and -4′, respectively (27, 28). Besides this Man-3, only α-linked mannose residues occur in the outer chains of the oligosaccharides. This is evidenced by the chemical shifts of the anomeric protons in combination with their $J_{1,2}$ coupling constants, each being about 1.5 Hz (compare (27, 28)). The relatively high field resonance position of H-1 of Man-4′ (δ 4.882), in conjunction

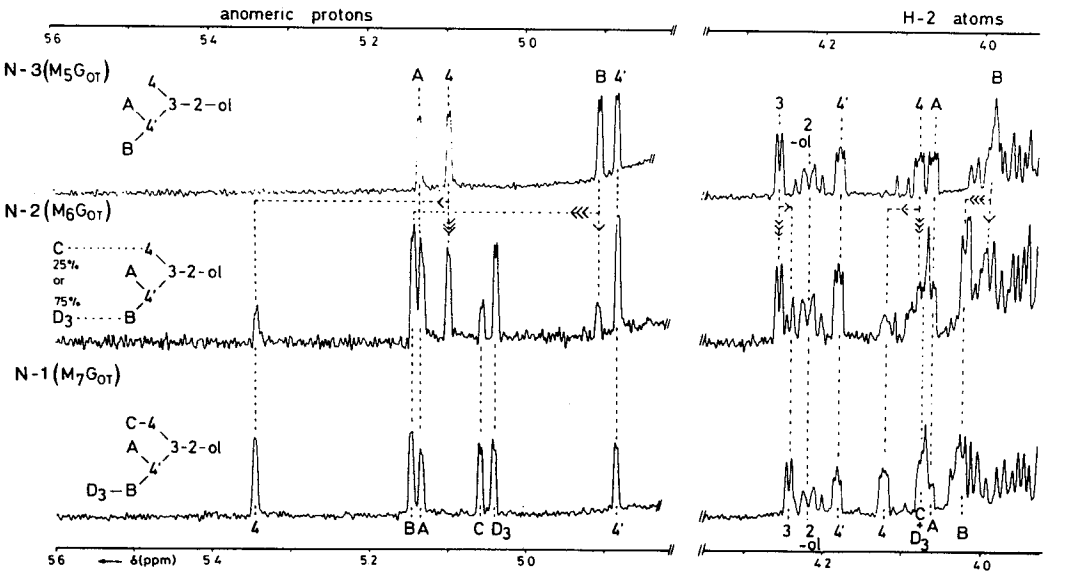

FIG. 6. Structural-reporter group (H-1 and H-2) regions of the resolution-enhanced 500-MHz ^{1}H NMR spectra of the neutral oligosaccharide fractions N-1, N-2, and N-3 obtained from porcine spleen cathepsin D in D_2O at 27°C. The numbers and letters in the spectra refer to the corresponding residues in the structures (see also Fig. 9). The subscript OT indicates that traces of $NaB[^3H]_4$-reduced oligosaccharides were included in the samples.

with the relatively downfield position of its H-2 (δ 4.180), is typical for a disubstitution of this residue at C-3 and C-6, by Man-A and -B, respectively (26, 27, 29–33). These similarities in the spectra of N-1, N-2, and N-3 indicate that the oligosaccharides have in common the

$$\underset{A}{\text{Man}\alpha 1} \rightarrow 3[\underset{B}{\text{Man}\alpha 1} \rightarrow 6]\underset{4'}{\text{Man}\alpha 1} \rightarrow$$

$$6[\underset{4}{\text{Man}\alpha 1} \rightarrow 3]\underset{3}{\text{Man}\beta 1} \rightarrow 4\underset{2}{\text{GlcNAcol}}$$

moiety, as usual for oligomannoside-type carbohydrate structures.

Oligosaccharide N-3 can readily be identified as the above hexasaccharide–alditol. The spectrum (Fig. 6, upper trace) contains four H-1 signals in the α-anomeric region. The assignments, given in Table II and Fig. 6, are based upon comparison with appropriate, oligomannoside-type reference compounds (26, 27, 29–33). The chemical shifts for the H-1 atoms of Man-4, -A, and -B each point to the terminal, nonreducing character of the residues concerned. Careful comparison of chemical shift data for N-3 with those for the corresponding glyco-asparagine, $Man_5GlcNAc_2Asn$, isolated from the urine of a patient with Gaucher's disease (29), or similar oligosaccharides (30) ending in GlcNAc-2, reveals that the H-1 and H-2 chemical shifts for Man-4′ and in particular those for Man-A show significant differences. These deviations demonstrate the apparent sensitivity of these chemical shifts to reduction of GlcNAc-2. This observation is another argument in favor of the close proximity of these residues to the anomeric center of GlcNAc-2. This conformational aspect has been previously proposed by us, on the basis of the anomerization effect shown by the aforementioned signals in the spectra of reducing oligosaccharides of the oligomannoside type (27, 30, 32).

Like N-3, oligosaccharide N-1 is a homogeneous oligosaccharide. The 500-MHz ^{1}H NMR spectrum of this sample (Fig. 6, lower trace) shows six α-anomeric signals of equal intensity. As compared to N-3, two additional H-1 signals are observable at $\delta \cong 5.05$. This points to the presence of two terminal α-1 → 2-linked Man residues (26, 27, 30–33). These can be localized in the different branches by tracing the shift effects which they induce upon the anomeric signals of the substituted Man residues (27). Going from N-3 to N-1, both the H-1 signal of Man-4 as well as that of Man-B have undergone a shift by $\Delta\delta \cong 0.25$ ppm (see Table II), whereas the chemical shifts of the other H-1 signals remain unaltered. Also, the H-2 signals of 4 and B show a significant downfield shift ($\Delta\delta \cong 0.04$ ppm). These effects indicate that Man-4 and Man-B in N-1 bear an α-1 → 2-linked substituent, designated Man-C and Man-D_3, respectively. The assignment of the H-1 signals of Man-C and Man-D_3 is based on data for reference oligosaccharides of the oligomannoside type (30, 33). It should be noted that the significant shift effect on H-2 of Man-3 is caused by the attachment of Man-C to Man-4 (compare (27, 30, 33)). The set of reporter-group chemical shifts for the octasaccharide–alditol N-1 is essentially in accord with that for $Man_7^*GlcNAc_2Asn$, obtained from IgM of a patient with Waldenström's macroglobulinemia (33).

The anomeric region of the ^{1}H NMR spectrum of N-2 shows three sets of signals, which can be divided according to their relative intensities (1.00:0.75:0.25). This indicates already that a mixture of (at least) two oligosaccharides is involved. The signals with relatively highest intensity (1.00) are those at δ 4.881 and 5.133, attributed to H-1 of Man-4′ and Man-A, respectively (compare N-1 and N-3). This implies that the components of mixture N-2 neither deviate from each other with respect to the disubstitution of Man-4′, nor as to the terminal character of Man-A. The signals with medium relative intensity (0.75) are those at δ 5.037, 5.099, and 5.141. These can be assigned to the H-1 atoms of terminal Man-D_3, terminal Man-4, and 1 → 2-substituted Man-B, respectively (compare to N-1). Therefore, the major component (75%) of mixture N-2, designated N-2a, is a heptasaccharide–alditol, which can be considered to be an extension of N-3 with Man-D_3 in α1 → 2 linkage to Man-B. The

signals of relatively lowest intensity (0.25) are those at δ 4.906, 5.053, and 5.343. These can be assigned to the H-1 atoms of terminal Man-B, terminal Man-C, and 1 → 2-substituted Man-4, respectively. Thus, the minor component (25%) of this mixture, designated N-2b, is a positional isomer of N-2a. It deviated from the main product only in the location of the terminal α1 → 2-linked Man residue: in N-2b, it is Man-C linked to Man-4, while in N-2a it is Man-D_3 linked to Man-B. The positions and relative intensities of the signals in the Man H-2 region of the spectrum (see Fig. 6 and Table II) are in complete accordance with this interpretation. The peripheral part of the structure of N-2b is identical to that of $Man_6GlcNAc_2Asn$ derived from ovalbumin (23, 27). The subtle differences between the ^1H chemical shifts for these two compounds are exclusively attributable to the difference in conformation at the potentially reducing end of GlcNAc-2 in both.

The present study demonstrates that reduced oligosaccharides of oligomannoside type ending in GlcNAc-2ol, are excellent alternatives for glycopeptides and reducing oligosaccharides used so far (26, 27, 29, 33) for structural investigations by ^1H NMR spectroscopy. Recently, we described the ^1H NMR parameters for a series of reduced oligosaccharides, also derived from asparagine-linked carbohydrate chains, but ending in

$$\begin{matrix} GlcNAc\beta1 \rightarrow 4GlcNAcol \\ 2 \qquad\qquad 1 \end{matrix}$$

(34). Comparing the ^1H NMR parameters of the reporter groups of the reduced GlcNAc residues, it appears that the H-2 resonance has the same position and shape for both 1ol and 2ol; however, the positions of the *N*-acetyl signals differ significantly (δ 2.059 for GlcNAc-2ol against $\delta \cong 2.054$ for GlcNAc-1ol).

Characterization of acidic oligosaccharides. To determine whether the net negative charge of fraction 2 (Fig. 3) was due to the presence of phosphate moieties, the fraction was treated with *Escherichia coli* alkaline phosphatase or was subjected to mild acid hydrolysis (pH 2.0, 100°C, 30 min) followed by alkaline phosphatase treatment. As shown in Fig. 7B, a series of peaks were generated after digestion with alkaline phosphatase corresponding to $Man_9GlcNAcol$ to $Man_5GlcNAcol$, which were, in turn, converted into a single peak comigrating with ManGlcNAcol by digestion with jack bean α-mannosidase (Fig. 7C). The dramatic shift in elution position following dephosphorylation, shown in Fig. 7B, is consistent with the sensitivity of the chromatographic system to charge. The results indicated that cathepsin D from porcine spleen contained phosphate residues in monoester linkage, most likely as mannose 6-phosphate, on a family of oligomannoside-type oligosaccharides which consisted of $Man_9GlcNAcol$, $Man_8GlcNAcol$, $Man_7GlcNAcol$, $Man_6GlcNAcol$, and $Man_5GlcNAcol$ in the ratio of 8:10:41:36:5. The content of the phosphorylated oligosaccharides all together amounted to 3.4% of the oligosaccharides released by endo-β-*N*-acetylglucosaminidase H. Mild acid hydrolysis of the alkaline phosphatase-resistant fraction (fraction I in Fig. 7B) followed by redigestion with alkaline phosphatase did not yield any change in the elution profile, suggesting that none of the phosphate residues in cathepsin D was present in diester linkage like GlcNAc-P-mannose (35–37). It should be noted that ^3H radioactivity recovered in fraction 1 in Fig. 7B seemed to be attributable to contaminating peptides rather than to oligosaccharides, because, after hydrolysis in 4 M HCl at 100°C for 4 h, no GlcNAcol could be detected in the fractions by paper chromatography using solvent II.

Interaction of cathepsin D with liver mannan-binding protein. Binding ability of cathepsin D to the mannan-binding protein isolated from rabbit livers was studied using cathepsin D as a possible inhibitor to the binding between the binding protein and a neoglycoprotein, $GlcNAc_{43}$–bovine serum albumin. As shown in Fig. 8, Scatchard plots of the binding data indicated that cathepsin D is a competitive inhibitor to the binding. The K_i value was estimated as 4.5×10^{-6} M, while the K_d value for $GlcNAc_{43}$–bovine serum albumin was 1.8×10^{-9} M.

DISCUSSION

The structures of the neutral oligosaccharides of cathepsin D from porcine spleen elucidated ultimately by the analysis with 500-MHz ^1H NMR spectroscopy in this study were identical with those recently proposed by Takahashi *et al.* (20), except for the presence of two isomeric oligosaccharides containing six mannoses. Of note in this concern is the fact that the ^1H NMR spectra presented by Takahashi *et al.* (20) were also interpretable as showing the occurrence of the isomer. However, complete separation of the oligosaccharides by gel permeation chromatography according to size prior to ^1H NMR analysis with high resolution were needed to come to the conclusion that the minor signals represent the occurrence of distinct isomeric components. On the other hand, Takahashi *et al.* (20) found an oligosaccharide containing three mannoses in considerable amounts (13%) which we could not detect. Reasons for the discrepancy are not clear, but it may be associated with the differences in the methods for the isolation of the oligosaccharide units. Endo-β-*N*-acetylglucosaminidase H is known to release oligosaccharides from a variety of oligomannoside-type glycopeptides, but the rate of reaction varies depending upon the length of the mannose units (23–25). Therefore, we could not eliminate the possibility that an oligosaccharide with three mannoses which is rather resistant to the enzyme was not released and subsequently lost during the process of high-voltage paper electrophoresis; the recovery of this step as measured by orcinol reaction was 80% which could be accounted for by the loss of a minor component. The relative abundance of premature oligosaccharides ($Man_7GlcNAcol$ and $Man_6GlcNAcol$) in this study is in contrast with the relative abundance of further processed oligosaccharides ($Man_5GlcNAcol$, oligosaccharides containing galactose or a third *N*-acetylglucosamine) in the study of Takahashi *et al.* (20), though reasons for the discrepancy are not currently clear. As for the differences in phosphate contents between the two studies (0.16 versus <0.02 mol/mol), it should be noted that, in this study, throughout the isolation procedures 1 mM Na_2HPO_4 was included to protect cathepsin D from phosphatase digestion and phosphate content was estimated by a specific and sensitive method.

In view of the current concept that asparagine-linked oligosaccharides have a common biosynthetic origin, $Man_9GlcNAc_2$ (38), a family of oligomannoside-type oligosaccharides of cathepsin D (encircled by dotted lines in Fig. 9) may represent the sequence of events in the biosynthetic pathway leading to the final mature form of $Man_5GlcNAc_2$. $Man_7GlcNAc_2$ occurred as a single isomer suggesting that the Man-D_2 residue was removed first, followed by Man-D_1. In the next step, the pathway was divided into two routes giving rise to two isomeric $Man_6GlcNAc_2$. The major trimming sequence of cathepsin D ($D_2 \rightarrow D_1 \rightarrow C \rightarrow D_3$) is unique as far as we know. The presence of the isomeric $Man_6GlcNAc_2$ (with Man-D_3) was reported in urine samples of mannosidosis (30, 39). In this case, however, all the variety of isomeric oligosaccharides which could arise from $Man_9GlcNAc_2$ occurred; hence the biosynthetic significance of the isomer is not clear. The minor sequence ($D_2 \rightarrow D_1 \rightarrow D_3 \rightarrow C$) is identical to what is known of oligosaccharides at Asn_{563} of IgM (33, 40) and of cellular glycoproteins of clone 15 B CHO cells (41). The processing of $D_1 \rightarrow D_3 \rightarrow C \rightarrow D_2$ which is a preferred sequence in the oligosaccharides at Asn_{402} of IgM (40) does not appear to be operative in cathepsin D.

Cathepsin D under study consisted of a heavy and a light chain, indicating the enzyme to be comprised almost exclusively of a mature form with regard to the biosynthetic processing of the polypeptide portion (18, 19). The proteolytic cleavage which generates the two-chain form from a parental single chain form seems to occur in the lysosomal compartment (18, 19, 42). Therefore, it may be concluded that the presence of a series of oligomannoside-type oligosaccharides with various lengths, which result from incomplete trimming of $Man_9GlcNAc_2$, does not disturb the normal sorting-out process of the enzyme from the

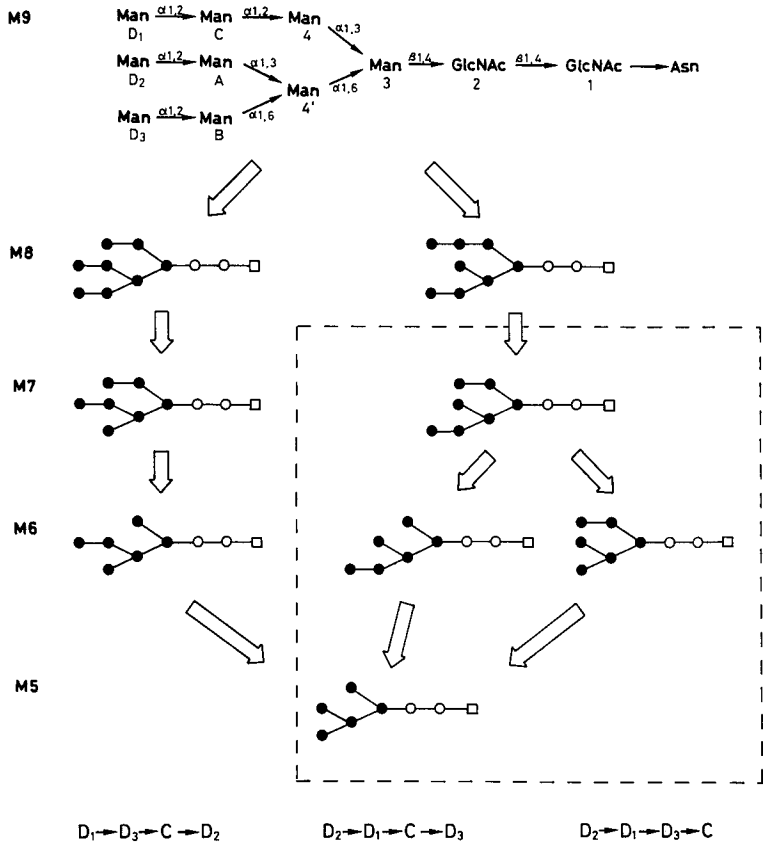

FIG. 9. Scheme for the processing of oligomannoside-type oligosaccharides of cathepsin D. The oligosaccharide structures of cathepsin D were encircled by dotted lines. The symbols are ●, mannose; ○, *N*-acetylglucosamine; □, asparagine.

site of biosynthesis to lysosomes. This is consistent with the results on β-glucuronidase from human spleen, in which $Man_9GlcNAc_2$ is the prevailing oligosaccharide (35, 43) and on α-mannosidase from porcine kidney (21). In contrast, β-glucuronidase from rat liver lysosomes is comprised only of $Man_5GlcNAc_2$ (44). With regard to the size of oligomannoside structures, it should be noted that α-mannosidase present in lysosomes can hardly hydrolyze oligomannoside structures on glycoproteins (45). Therefore, it is unlikely that heterogeniety in the size of oligomannoside structures on lysosomal enzymes is generated during the period of storage in lysosomes.

Scantiness of phosphorylated oligosaccharides in cathepsin D, representing 3.4% of the oligosaccharides released by endo-β-*N*-acetylglucosaminidase H, is in contrast with the results of the studies on the biosynthetic intermediates of lysosomal enzymes (5, 36, 37), wherein as much as 30–50% of the oligosaccharides released by endo-β-*N*-acetylglucosaminidase H were phosphorylated. On the other hand, the phosphorylated oligosaccharide content of the mature lysosomal enzymes in tissues is generally low except for those from cultured fibroblasts (35, 43, 44). A unique property of cathepsin D is the lack of a phosphodiester which is predominant in most of the phosphorylated oligosaccharides (35–37). All of the phosphorylated oligosaccharides in cathepsin D are of the oligomannoside type and are more heterogeneous in size and larger than the neutral oligosaccharides.

Our cathepsin D and β-glucuronidase

from rat livers (44) have in common the absence of complex-type oligosaccharides; however, this is in contrast to the situation with the following lysosomal enzymes: β-glucuronidase from human spleen (43) and from mouse macrophage-like cells (37), cathepsin D and β-hexosaminidase from human fibroblasts (46), and α-mannosidase from porcine kidney (21).

Despite the variations and heterogeneities discussed above, the lysosomal enzymes in the lysosomal compartment have a common property of being comprised predominantly of mannose-terminated oligosaccharides, mostly in the form of the oligomannoside type. This structural feature is in contrast to that of serum glycoproteins which bear complex-type oligosaccharides with a few exceptions (47). Because of this characteristic, lysosomal enzymes appear to be recognized by endocytosis receptors on hepatic sinusoidal cells and alveolar macrophages, and also by a hepatic lectin specific for mannose and *N*-acetylglucosamine (mannan-binding protein). In fact, cathepsin D was shown to be a good ligand to the mannan-binding protein isolated from rabbit livers. The binding protein, which is localized predominantly in microsomes, may be associated with the transport of biosynthetic intermediates of glycoproteins including lysosomal enzymes in the liver (11).

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MATERIALS AND METHODS

Materials.
Bio-Gel P-2 (minus 400 mesh), AG 50W-X4 (100-200 mesh), AG 1-X2 (100-200 mesh) and AG 3 (100-200 mesh) were purchased from Bio-Rad Laboratories. Sephadex G-25 (fine) and G-50 (fine) were from Pharmacia. $NaB[^3H]_4$ (469 mCi/mmol) was a product of the Radiochemical Centre, Amersham, England. [1-^{14}C] Acetic anhydride (28 mCi/mmol) was obtained from New England Nuclear. Man$_3$GlcNAcol was prepared from Taka-amylase A as described previously (S1), from which Manβ1→4GlcNAcol was prepared by exhaustive digestion with jack bean α-mannosidase. $GlcNAc_4$-bovine serum albumin was kindly provided by Dr. Yuan Chuan Lee of Johns Hopkins University, Baltimore. Pepstatin A was obtained from Protein Research Foundation, Osaka, Japan. Pepstatin-aminohexyl-Sepharose 4B gels were prepared according to the method of Huang et al. (S2). Fresh porcine spleens were obtained at a local slaughter-house and kept frozen at -70°C.

Enzymes.
Endo-β-N-acetylglucosaminidase H from Streptomyces griseus (S3-S5) was obtained from Seikagaku Kogyo Co., Japan. Pronase P from Kaken Kagaku Co., Japan and alkaline phosphatase from Escherichia coli from Sigma. Purified α1→2-specific mannosidase from Aspergillus saitoi (S6,S7) was kindly provided by Dr. Eiji Ichishima, Tokyo Noko University, Tokyo, Japan. Jack bean α-mannosidase was prepared according to Li and Li (S8) and β-mannosidase from a snail, Achatina fulica, according to Sugahara et al. (S9).

Purification of cathepsin D from porcine spleen.
Cathepsin D was purified from porcine spleen according to the method of Huang et al. (S2) with minor modifications. Frozen and thawed spleens were homogenized in a Waring blendor with 1 mM Na_2HPO_4 and the homogenate was centrifuged at 10,000 rpm for 30 min. Throughout the following isolation procedures, 1 mM Na_2HPO_4 was included in all the solutions and buffers to protect cathepsin D from phosphatase digestion. To the supernatant, solid ammonium sulfate was added to give a 70% saturation. The precipitate was collected and dialyzed against distilled water. The pH of the solution was adjusted to 3.7 with 6M HCl and the precipitate formed was removed by centrifugation. To the acid supernatant, solid ammonium sulfate was added to give a 70% saturation. The precipitate was dialyzed against 0.05 M sodium phosphate buffer at pH 7.4 and then subjected to a DEAE-Sephadex A-25 column which had been equilibrated with the same buffer. Unadsorbed fractions, in which cathepsin D was recovered, were adjusted to pH 3.5 prior to mixing with pepstatinyl-aminohexyl-Sepharose gel. Cathepsin D bound to the gel was specifically eluted with 0.05 M borate buffer, pH 8.5 containing 0.2 M NaCl. By these procedures, approximately 36 mg of purified cathepsin D with a specific activity of 16 units per mg of protein was recovered from 500 g of frozen spleen with a 27% recovery from the homogenate with a 250-fold purification. Upon polyacrylamide gel electrophoresis at pH 9.45 (Fig. 1), the purified sample showed one major and 3-4 minor bands, corresponding to cathepsin D isozymes previously shown by Huang et al. using isoelectric focusing (S2). Upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the purified sample gave rise to two major bands, corresponding to the heavy (Mr=35,000) and light (Mr=15,000) chains as well as two minor bands with high molecular weight, the results being consistent with those shown by Huang et al. (S2) (data not shown).

Preparation of glycopeptides.
Purified cathepsin D (96.4 mg of protein), which had been denatured in boiling water, was exhaustively digested with pronase P (2.0 mg) in 7 ml of 0.2 M borate buffer at pH 8.0, containing 10 mM $CaCl_2$, 0.2 M glucose-6-phosphate and 0.1 M 2-mercaptoethanol under a toluene atmosphere at 37°C for 116 h. Glucose-6-phosphate and 2-mercaptoethanol were included to inhibit phosphatase and phosphodiesterase detectable in pronase P employed here. After 48, 72 and 96 h, additional pronase P (2.0, 0.6 and 0.6 mg) and 2-mercaptoethanol (30, 10 and 10 µl were added, respectively. After centrifugation of the digest, the supernatant was applied to a Sephadex G-50 column (1.2 x 118 cm), followed by a Sephadex G-25 column (1.4 x 115 cm) using 0.05 M pyridine-acetic acid at pH 5.0 as a developing buffer. Fractions positive to orcinol-H_2SO_4 reaction were collected at each step of chromatography.

Endo-β-N-acetylglucosaminidase H digestion of glycopeptides.
Cathepsin D glycopeptides (15.5 µmol of mannose) were digested with endo-β-N-acetylglucosaminidase H (0.1 unit) in 200 µl of 50 mM citrate buffer, pH 5.0, at 37°C for 69 h under a toluene atmosphere, with the enzyme (0.1 unit) added newly after 24 and 48 h. The digestion was monitored by measuring reducing activity by the method of Park and Johnson (S10) and terminated by boiling the mixture for 2 min. The digest was applied to a Sephadex G-25 column (1.4 x 115 cm) to separate oligosaccharides from N-acetylglucosaminyl peptides.

Table I

CARBOHYDRATE CONTENT OF CATHEPSIN D

Carbohydrate	mg/100 mg of protein	mol/mol of protein
Mannose	3.3	9.2
Galactose	n.d.	---
Glucose	n.d.	---
Fucose	< 0.1	---
Glucosamine	1.4	3.8
Galactosamine	n.d.	---
Sialic acid	n.d.	---
Mannose-6-phosphate	0.08	0.16

Note. Analyses were carried out as described under "Materials and Methods". No correction was made for loss during hydrolysis. n.d. means not detected.

Fig. 1 Polyacrylamide gel electrophoresis of cathepsin D from porcine spleen. Purified cathepsin D (15 µg) was applied to a 7.5% acrylamide gel. Electrophoresis was carried out using "System A" (S13) (pH 9.54). Protein bands were stained with Coomassie brilliant blue G-250 in 10% trichloroacetic acid. Arrow denotes the migration of marker dye (bromophenol blue).

Reduction of oligosaccharides.
A portion of the oligosaccharides (0.39 µmol of mannose) was reduced in 0.5 ml of 0.1 M borate buffer, pH 8.0, at 30°C with 1 mCi of $NaB[^3H]_4$ for 2 h and then with 5.3 µmol of $NaBH_4$ for 3 h. The reaction was terminated by the addition of 200 µl of acetone and the mixture was adjusted to pH 5 with acetic acid prior to repeated evaporation with methanol to remove boric acid. Unlabeled reduced oligosaccharides were prepared by treating the oligosaccharides (15 µmol of mannose) with an excess of $NaBH_4$ under otherwise the same conditions. The reduced oligosaccharides were cleaned up by gel filtration on a column of Sephadex G-25 (1.4 x 115 cm).

Acetylation of peptides.
Since the reduced oligosaccharide fraction was contaminated with significant amounts of peptides (0.5 mol per mol of mannose) as estimated by fluorescamine reaction according to Nakai et al. (S11), NH_2-termini of the peptides were labeled with [1-^{14}C]acetic anhydride to monitor peptides in the

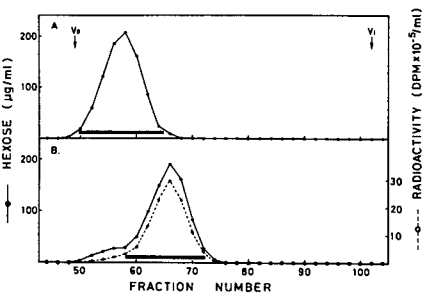

Fig. 2 Sephadex G-25 column chromatography of the pronase digest of cathepsin D and of oligosaccharides released from this digest by endo-β-N-acetylglucosaminidase H. Pronase digest of cathepsin D, which had been passed over a Sephadex G-50 column, was applied to a Sephadex G-25 (fine) column (1.4 x 115 cm)(panel A). The glycopeptide fraction in panel A (15.5 µmol of mannose) was digested with endo-β-N-acetylglucosaminidase H and the released oligosaccharides were reduced with $NaB[^3H]_4$, and applied to the same Sephadex G-25 column (panel B). Fractions of 1.7 ml were collected. Aliquots were used for the determination of hexose (-●-) and radioactivity (-○-). The fractions were pooled as indicated by bars.

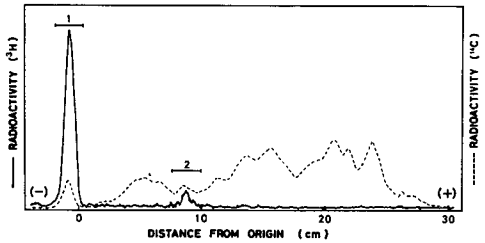

Fig. 3 Electrophorogram of the oligosaccharide fraction obtained from the pronase digest of cathepsin D. High-voltage paper electrophoresis was performed at pH 5.4. The ^{3}H-labeled oligosaccharide fraction (5.2 x 10^5 dpm) and the ^{14}C-labeled oligosaccharide fraction (1.2 x 10^5 dpm) were applied in a separate lane on the same sheet. Each radiochromatogram, monitored by a radiochromatogram scanner, was superimposed on a single chart.

following experiments. Thus, to the reduced oligosaccharide fraction (0.7 µmol of peptide and 1.5 µmol of mannose) in 0.3 ml of 0.5% $NaHCO_3$, 0.9 µmol of [1-^{14}C]acetic anhydride (25 µCi) was added. After 1 h at room temperature, 12 µmol of unlabeled acetic anhydride was added and the reaction allowed to proceed for 1 additional hour. The products were isolated by gel filtration on a column of Sephadex G-25 (1.4 x 115 cm).

Paper electrophoresis and paper chromatography.
High voltage paper electrophoresis was performed on Whatman 3MM paper using pyridine/acetic acid/H_2O (3/1/387, V/V), pH 5.4, as a buffer at 75 V/cm for 45 min. Descending paper chromatography was performed with Toyo No. 51A paper using the following solvent systems: I, ethyl acetate/pyridine/acetic acid/H_2O (5/5/1/3, V/V); II, nitromethane/ethanol/acetic acid/sat. boric acid (8/1/1/1, V/V).

Gel permeation chromatography.
Bio-Gel P-2 column chromatography was performed at 55°C using a column equipped with a water jacket. For analytical purpose, a column (1.8 x 190 cm) was eluted with H_2O and for preparative purpose, a column (1.5 x 190 cm) was equilibrated and eluted with 50 mM pyridine-acetic acid, pH 5.0. Calibration of the column was carried out with glucose oligomers and oligosaccharides of known structure, e.g., $Man_5GlcNAcol$, and Manβ1→4GlcNAcol.

Enzymatic digestion of oligosaccharides.
All digestions were performed at 37°C under a toluene atmosphere and terminated by heating the incubation mixture in boiling water for 2 min. Jack bean α-mannosidase digestion was performed with 1.0 unit of the enzyme in 100 µl of 0.6 M acetate buffer, pH 4.5, containing 0.24 mM $ZnCl_2$ for 40 h. Aspergillus saitoi α-mannosidase digestion was performed with 1.5 µg of the enzyme in 40 µl of 0.16 M acetate buffer, pH 5.0, for 7 days. Snail β-mannosidase digestion was performed with 0.225 unit of the enzyme in 50 µl of 0.5 M acetate buffer, pH 4.5, for 4 days. E. coli alkaline phosphatase digestion was performed with 0.4 unit of the enzyme in 40 µl of 0.25 M Tris/HCl buffer, pH 8.0, for 48 h. The digests were analyzed by gel permeation chromatography on a Bio-Gel P-2 column (1.8 x 190 cm).

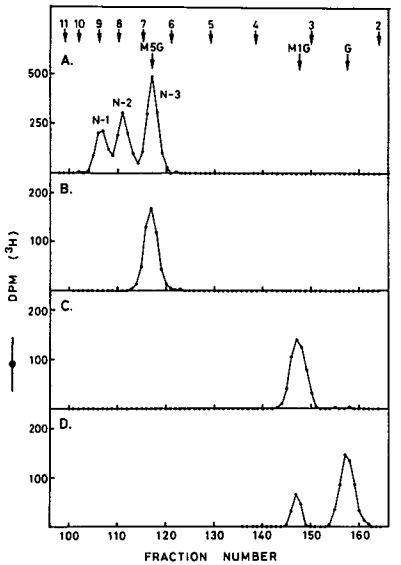

Fig. 4 Gel permeation chromatography of the neutral oligosaccharides and their exoglycosidase digests. The radioactive oligosaccharides were subjected to a Bio-Gel P-2 column (1.8 x 190 cm). Fractions of 1.9 ml were collected and aliquots thereof were used for monitoring radioactivity. A, the neutral oligosaccharides (Fig. 3) (1.8 x 10^5 dpm). B, α-1→2-specific mannosidase digest of A (1.5 x 10^4 dpm). C, jack bean α-mannosidase digest of B (1.7 x 10^4 dpm). D, β-mannosidase digest of C (9.0 x 10^3 dpm). The arrows denote the elution positions of glucose oligomers (numbers indicate the glucose unit) and authentic samples (M_5G, $Man_5GlcNAcol$; M_1G, ManGlcNAcol; G, GlcNAcol).

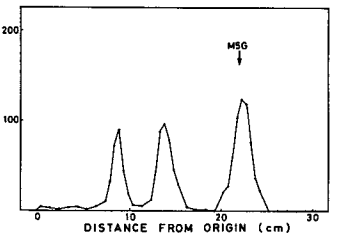

Fig. 5 Paper chromatography of the neutral oligosaccharides. The neutral oligosaccharides (Fig. 3) (3.0 x 10^4 dpm) were analyzed by descending paper chromatography developed for 14 days using Solvent I. The paper was cut into one-cm segments prior to measuring radioactivity.

Preparation of oligosaccharides for 500-MHz ^{1}H-NMR analysis.
The neutral oligosaccharide fraction isolated by high voltage paper electrophoresis (Fig. 3) was applied to an AG 1x2 (AcO^- form) column (0.5 x 5 cm) to remove contaminating peptides. Oligosaccharides recovered in the unadsorbed fraction were separated into $Man_7GlcNAcol$, $Man_6GlcNAcol$ and $Man_5GlcNAcol$ by a Bio-Gel P-2 column (1.5 x 190 cm). After rechromatography with the same column, each oligosaccharide solution was evaporated to dryness.

500-MHz ^{1}H-NMR spectroscopy.
Prior to NMR spectroscopic analysis, the neutral oligosaccharide fractions were repeatedly treated with D_2O at room temperature. After each exchange treatment, the materials were lyophilized. Finally, each sample was redissolved in 0.4 ml D_2O (99.96 atom% D, Aldrich, U.S.A.) for examination in a 5 mm-tube (528 PP, Wilmad, U.S.A.). 500-MHz ^{1}H-NMR spectroscopy was performed on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysics, Nijmegen University, The Netherlands), operating in the Fourier transform mode, and equipped with a Bruker Aspect 2000 computer. For further experimental details, see (S12). Resolution enhancement of the spectra was achieved by Lorrentzian to Gaussian transformation (S12). The probe temperature was 27°C, and was kept constant within 0.1°C. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ 2.225 in D_2O at 27°C), with an accuracy of 0.002 ppm.

Polyacrylamide gel electrophoresis.
Polyacrylamide gel electrophoresis was performed using System A (pH 9.45) as described by Rodbard and Chrambach (S13).

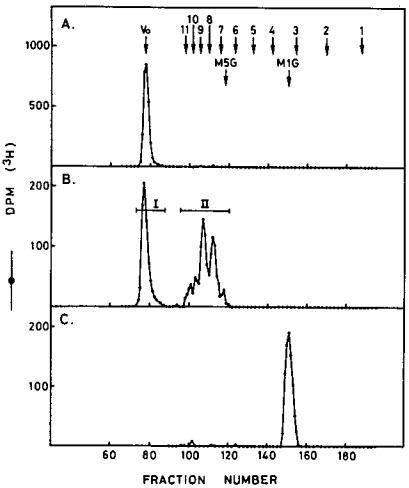

Fig. 7 Gel permeation chromatography of the acidic oligosaccharides and their alkaline phosphatase digest. Analytical conditions of radioactive sugars and arrows are the same as in Fig. 4. A, the acidic oligosaccharides (Fig. 3) (7.8 x 10^4 dpm). B, alkaline phosphatase digest of A (5.1 x 10^4 dpm). C, jack bean α-mannosidase digest of fraction II in B (2.3 x 10^4 dpm).

Table II

^{1}H CHEMICAL SHIFTS OF STRUCTURAL-REPORTER GROUPS OF CONSTITUENT MONOSACCHARIDES FOR THREE NEUTRAL OLIGOSACCHARIDE FRACTIONS OBTAINED FROM PORCINE SPLEEN CATHEPSIN D

Reporter group	Residue[a]	Chemical shift[b] in N-1	N-2a	N-2b	N-3
H-1 of	4	5.343	5.099	5.343	5.096
	4'	4.883	4.881	4.881	4.882
	A	5.132	5.133	5.133	5.133
	B	5.145	5.141	4.906	4.904
	C	5.057	-	5.053	-
	D_3	5.039	5.037	-	-
H-2 of	2ol	4.214	4.218	4.218	4.218
	3	4.241	4.253	4.243	4.256
	4	4.120	4.078	4.119	4.079
	4'	4.180	4.180	4.180	4.178
	A	4.063	4.062	4.062	4.061
	B	4.023	4.016	3.992	3.98
	C	4.068	-	4.067	-
	D_3	4.068	4.067	-	-
NAc of	2ol	2.059	2.059	2.059	2.059

[a] For numbering of monosaccharide residues and types of linkages, see Fig. 9.

[b] Chemical shifts were acquired at 500 MHz; they are given in ppm downfield from DSS for D_2O solutions at 27°C. At this probe temperature, the H-1 signal of Man-3 is hidden under the residual HOD line at $\delta \approx 4.78$.

Analytical methods.

Hexose was determined by the orcinol-H_2SO_4 method of Hewitt (S14). Sialic acid was determined by the resorcinol method of Jourdian et al. (S15). Hexosamine was determined with a Hitachi amino acid analyzer 835, after hydrolysis in 6 M HCl at 100°C for 16 h. Neutral sugars were separated and estimated by mass-fragmentography after conversion into alditol acetates as described previously (S16). Mannose-6-phosphate was estimated enzymatically according to the methods of Gawehn (S17), and Asikin and Koeppe (S18) with minor modifications. Cathepsin D (100 µg) was hydrolyzed in 400 µl of 2 M trifluoroacetic acid at 100°C for 6 h. After evaporation to dryness, the hydrolysate was taken up in 3.5 ml of 0.05 M Tris/HCl, pH 7.6 containing 3 mM $MgCl_2$. To 1 ml of the solution, 10 µl of 50 µM NADP, 5 µl of glucose-6-phosphate dehydrogenase (350 units/ml), 2 µl of phosphoglucose isomerase (700 units/ml) and 5 µl of phosphomannose isomerase (120 units/ml) were added. After incubation at 25°C for 70 min, NADPH produced was measured fluorometrically at the excitation wavelength of 340 nm and the emission wavelength of 455 nm using a Hitachi fluorescence spectrophotometer, model 650-10S. Mannose-6-phosphate was used as a standard and the blank value was obtained by incubating the sample under the same conditions except for the omission of phosphomannose isomerase or glucose-6-phosphate dehydrogenase. Radioactivity was measured with a Beckman liquid scintillation spectrophotometer model LS 7500, using a toluene-Triton X-100 scintillation mixture (S19). Radiochromatogram scanning was performed with an Aloka radiochromatogram scanner, model JPC-213.

Estimation of Ki for cathepsin D to the mannan-binding protein.

The assay for the mannan-binding protein was carried out as described previously (S20), in the presence of 0.8 µg of the purified binding protein from rabbit livers and 33 to 250 ng of ^{125}I-labeled GlcNAc$_{4,3}$-bovine serum albumin with or without cathepsin D (114 µg).

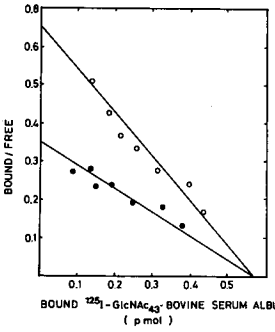

Fig. 8 Interaction of cathepsin D with liver mannan-binding protein. The binding data between the mannan-binding protein from rabbit liver and the various amounts of ^{125}I-GlcNAc$_{4,3}$-bovine serum albumin from 33 to 250 ng in the presence (●) or the absence (○) of cathepsin D were analyzed by Scatchard plots.

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