This is to certify that the

dissertation entitled

Purification and characterization of caprine and bovine β-mannosidases. Characterization of a cDNA cloned with anti-β-mannosidase serum

presented by

BRYCE SOPHER.

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

Margaret Jones
Major professor

Date 16 March 1992

# PURIFICATION AND CHARACTERIZATION OF CAPRINE AND BOVINE ß-MANNOSIDASES. CHARACTERIZATION OF A cDNA CLONED WITH ANTI-ß-MANNOSIDASE SERUM

By

Bryce Sopher

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Interdepartmental Doctoral Program

1992

## ABSTRACT

PURIFICATION AND CHARACTERIZATION OF CAPRINE AND BOVINE ß-MANNOSIDASES. CHARACTERIZATION OF A cDNA CLONED WITH ANTI-ß-MANNOSIDASE SERUM

By

Bryce Sopher

Molecular linkage analysis of a goat pedigree shows that a guinea pig cDNA of interest, obtained from Glyn Dawson at the University of Chicago, does not consistently segregate with the caprine ß-mannosidosis disease gene. These findings demonstrate that the cloned guinea pig gene does not correspond to the caprine ß-mannosidosis disease gene and strongly suggest that the gene does not encode ß-mannosidase.

Goat ß-mannosidase was purified 120,000 fold in 26% yield from kidney using Concanavalin A-Sepharose, immunoaffinity and cation-exchange chromatography. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining, the immunoaffinity purified enzyme preparation consists of three prominent peptides (80, 90 and 100 kDa). The 90 and 100 kDa peptides are both associated with ß-mannosidase activity while the 80 kDa peptide is not. The 90 and 100 kDa peptides both react with anti-ß-mannosidase monoclonal antibodies and produce similar electrophoretic peptide patterns when subjected to limited proteolysis. The 90 and 100 kDa peptides were not detected in goat kidney tissues from animals with ß-mannosidase deficiency while the abundance of the 80 kDa peptide appeared normal.

Immunoaffinity purified bovine ß-mannosidase (from kidney

tissue) also consists of three peptides (80, 100 and 110 kDa). The bovine 80 kDa peptide is immunologically related to the goat 80 kDa peptide and the 100 and 110 kDa peptides are immunologically related to the goat 90 and 100 kDa peptides. Removal of N-linked carbohydrate reduces the bovine (100 and 110 kDa) and goat (90 and 100 kDa) peptides to 86 and 91 kDa. Kidney tissue from calves with ß-mannosidase deficiency does not contain detectable quantities of the 80, 100 or 110 kDa peptides.

These findings suggest that the bovine (100 and 110 kDa) and the goat (90 and 100 kDa) peptides represent two related forms of ß-mannosidase. The apparent absence of these peptides in tissues from animals with ß-mannosidase deficiency further substantiates their identity and begins to define the molecular basis of the goat and bovine mutations.

**ACKNOWLEDGEMENTS**

I sincerely thank both Dr. Karen Friderici and Dr. Margaret Jones for their guidance, support and encouragement throughout my four years of research. I would also like to acknowledge Christine Traviss (my part-time co-worker) for her contributions to the protein characterization work. I also wish to thank Dr. Steven Triezenberg, Dr. John Wilson and Dr. Robert Hausinger for their work as members of my Ph.D. committee.

**TABLE OF CONTENTS**

## LIST OF TABLES

## LIST OF FIGURES

**Bovine ß-mannosidase: Purification, characterization and peptide sequence analysis**

# INTRODUCTION

Lysosomes are cytoplasmic organelles that contain several dozen different acid hydrolases which are collectively referred to as lysosomal enzymes. These enzymes are responsible for the degradation of large cellular molecules (nucleic acids, proteins, polysaccharides and lipids). The dysfunction of any one of these enzymes leads to the accumulation of undigested substrate(s) which over the course of time leads to a progressive increase in the number and size of lysosomes within the cell. The deficiency of each enzyme has its most marked effects on a characteristic set of cell types. It is generally accepted, in the absence of concrete evidence, that the presence of too many large lysosomes in a cell can lead to cellular dysfunction which in turn can lead to clinical manifestations. It is unclear, however, exactly what cellular functions may be affected and to what extent (Holtzman, 1989a).

## The Basic Steps of Lysosomal Enzyme Synthesis and Transport

As with secretory proteins, lysosomal proteins are synthesized on polysomes bound to the endoplasmic reticulum, inserted into the endoplasmic reticulum, amino terminal signal sequences are cleaved and a preassembled oligosaccharide (containing a branched chain of two N-acetylglucosamines, nine mannoses and three glucoses) is attached to the amino groups

of specific asparagines. As the enzymes traverse the endoplasmic reticulum glucosidases remove all of the glucoses and one to three of the mannoses (von Figura and Hasilik, 1986). Up to this point the oligosaccharides are described as "high mannose".

Upon reaching the Golgi apparatus the carbohydrate moieties are subjected to additional processing. As the lysosomal protein passes through the Golgi apparatus some of the branches ("antennae") of the oligosaccharides will escape processing, others will receive a mannose-6-phosphate group and some will be subjected to extensive remodeling (Kornfeld and Mellman, 1989).

Phosphorylation of mannoses is unique to lysosomal enzymes and serves as a marker for targeting to the lysosome. The phosphate group is attached to the mannose in a two step process. First a phosphotransferase links a N-acetylglucosamine-1-phosphate via a phosphodiester bond to the hydroxyl on carbon-6 of one of the mannose residues. The three dimensional structure, common to lysosomal proteins, which specifically interacts with the phosphotransferase is slowly being resolved (Kornfeld, 1990; Baranski et al., 1990; Baranski et al., 1991). Following N-acetylglucosamine-1-phosphate attachment a phosphodiester glycosidase removes the N-acetylglucosamine, leaving the phosphate attached to the mannose. Generally, several of the mannoses in any given lysosomal protein will be phosphorylated in this way. The

phosphate blocks further processing of the antenna in the Golgi apparatus maintaining its "high mannose" nature (Holtzman, 1989b).

Similar to secretory proteins, some of the antennae which escape phosphorylation will be subjected to further processing events in the Golgi. The steps of this processing are as follows: mannosidases I and II trim the N-linked oligosaccharide chain down to two or three mannoses, N-acetylglucosamine transferase adds an N-acetylglucosamine to the antennae and then galactose and sialic acid are sequentially added by their corresponding transferases (von Figura and Hasilik, 1986). Antennae processed in this way are referred to as "complex".

The process of oligosaccharide remodeling allows one to group the carbohydrate antennae into three general classes (high mannose, high mannose/phosphorylated and complex). Because each N-linked carbohydrate moiety contains two to three antennae the carbohydrate moieties found on lysosomal proteins can be grouped into three classes: high mannose, complex and hybrid. Hybrid moieties are those in which one or more antennae are complex and one or more antennae are high mannose.

Sorting of soluble lysosomal proteins from other soluble secretory proteins in the trans-Golgi network is mediated by one of two different mannose-6-phosphate receptors (cation dependent and cation independent) (Dahms et al., 1989). The

most widely held view of lysosomal transport suggests that the mannose-6-phosphate receptors (localized in clathrin coated pits in the trans-Golgi network) localize lysosomal proteins to these pockets. Subsequently, these pockets bud off from the trans-Golgi network forming clathrin coated vesicles. Lysosomal proteins are transported in these vesicles to "prelysosomes" where the acidic environment facilitates their release from the receptor. Membrane associated lysosomal proteins do not require the mannose-6-phosphate signal for targeting (Rijnboutt et al., 1991; Waheed et al., 1988; Gottschalk et al., 1989). The presence of near normal levels of intra-lysosomal enzyme activities in many cell types in patients afflicted with I-cell disease (phosphotransferase deficiency) suggests that some cell types have another (as yet uncharacterized) mechanism of lysosomal transport (Tsuji et al., 1988). Basic issues such as: What exactly is a prelysosome? or How are clathrin coated vesicles targeted? are still unresolved.

## Lysosomal Processing of Lysosomal Enzymes

Early electrophoretic studies of lysosomal enzymes revealed considerable heterogeneity among the molecules of a given enzyme. Now many of the "isozymes" identified in such studies are known to correspond to different steps in the processing

and "maturation" of these molecules (Holtzman, 1989b). Proteolytic processing (as judged by pulse-chase labelling experiments) has been observed for all of the lysosomal enzymes evaluated (Skudlarek et al., 1984; Bergmann and Grabowski, 1989; Quon et al., 1989; Mahuran et al., 1988; DiCioccio and Mahoney, 1990; Taylor et al., 1990; Hoefsloot et al., 1990). These lysosomal proteolytic events can give rise to two or more associated peptide chains and/or two or more related peptides of varying size (Skudlarek et al., 1984; Clements et al., 1989; Deval et al., 1990). Analysis of the proteolytic processing regions suggests that these processing events are not carried out by specific "processing" proteases but rather are the result of limited proteolysis of accessible domains (Myerowitz and Neufeld, 1981; Clements et al., 1989; Hasilik and Neufeld, 1980; Frisch and Neufeld, 1981; Mahuran et al., 1988; Erickson, 1989). These proteolytic events can stretch out for hours or even days, as if the proteins are first attacked at particularly susceptible points and then are gradually nibbled down to a final, resistant form (Holtzman, 1989b).

The functional significance associated with these proteolytic processing events is unclear. Functionally the secreted "precursor" forms of lysosomal glycosidases are similar to the "processed" lysosomal forms (von Figura and Hasilik, 1986). This suggests that these maturation events may simply be a tolerated consequence of lysosomal coexistence.

Cathepsins on the other hand are activated by the first lysosomal cleavage event while subsequent cleavage events appear to have little or no functional consequences (Erickson, 1989). Whether the proteolytically removed peptides play a role in the targeting or folding of lysosomal enzymes is not yet known.

Glycosidic processing of the N-linked oligosaccharides attached to lysosomal enzymes seems to take a leisurely course similar to peptide maturation. All lysosomal enzymes evaluated have lost some of their carbohydrate in the lysosome (Neufeld, 1991).

The type and amount of carbohydrate associated with lysosomal enzymes is most often characterized by in vitro deglycosylation with Endoglycosidase H and N-Glycosidase F. Endoglycosidase H removes only the high mannose forms of oligosaccharides (those containing five or more mannoses and lacking terminal sialic acid or galactose residues). Therefore digestion with Endoglycosidase H allows one to estimate the amount of high mannose carbohydrate attached to a lysosomal enzyme. Since complex oligosaccharides usually contain only two to three mannoses, they can not be processed in the lysosome to a high mannose form. Therefore, digestion with Endoglycosidase H allows one to estimate the amount of N-linked oligosaccharide that escaped the additional processing (addition of galactose and sialic acid) in the mid and late Golgi compartments. Digestion with N-Glycosidase F

allows one to estimate the total amount of N-linked oligosaccharide. The difference between the N-Glycosidase F estimate and the Endoglycosidase H estimate of N-linked carbohydrate represents the oligosaccharides which were processed to complex types in the mid and late Golgi compartments and the high mannose oligosaccharides which were digested by lysosomal α-mannosidase to the extent that they contained four or fewer mannoses.

## Lysosomal Enzyme Stability

Lysosomal enzymes are relatively long-lived. Half lives of a day to several weeks are typical, and for some enzymes (such as the sulfatases) estimates are typically longer (Holtzman, 1989b). Several hypotheses have been put forth to explain how these degradative enzymes manage to coexist. One theory is that lysosomal enzymes have evolved with protease-resistant conformations. Another theory is that the mobility of lysosomal enzymes is restricted in some way, possibly by association with the lipid membrane or possibly by attachment to an intra-lysosomal matrix (assuming one exists). A third theory is that lysosomal enzymes exist in protective complexes within the lysosome ("circle your wagons theory").

There is no evidence that suggests that lysosomal enzymes have a unique protease-resistant conformation and there is

also no evidence that partially purified lysosomal enzymes are exceptionally resistant to proteolysis. With the exception of glucocerebrosidase there is little evidence that lysosomal enzymes are intimately associated with the lysosomal membrane. However, there is one well characterized example of a protective complex.

Neuraminidase, ß-galactosidase and a carboxypeptidase exist in the lysosome as a large (approximately 700 kDa) multienzymic complex (d'Azzo et al., 1982). The subunit composition and overall structure of the complex is slowly being resolved (Potier et al., 1990). Work leading to the characterization of this complex began with the characterization of an unusual combined deficiency of neuraminidase and ß-galactosidase. This disease was unique in the following respect: combined deficiency fibroblasts complemented both ß-galactosidase and neuraminidase deficient fibroblasts, suggesting the involvement of a third gene (Hoeksema et al., 1979). Characterization of the turnover time of ß-galactosidase in "combined deficiency" fibroblasts revealed that the half life of the enzyme was less than a day, whereas the half life of the enzyme in normal fibroblasts is approximately 10 days (van Diggelen et al., 1982). The identification and characterization of a glycoprotein in normal fibroblasts ("corrective factor"), capable of restoring the stability of ß-galactosidase, led to the cloning and characterization of the associated carboxypeptidase

("protector protein")(Galjart et al., 1988). Additional studies demonstrate that this carboxypeptidase is necessary for the multimerization and stabilization of both ß-galactosidase and neuraminidase in the lysosome (Potier et al., 1990).

Analogous complexes have not been described for other lysosomal enzymes. However, Hopwood and co-workers have noted that the heparan sulphate-degrading enzymes are inhibited by their products and by each other's substrate. They have interpreted these observations, which demonstrate the involvement of similar aglycone structures in binding and catalysis, to suggest that all of the sulphate-degrading enzymes operate in close proximity to each other, possibly as an enzyme complex (Bielicki et al., 1990).

## ß-Mannosidosis

N-linked oligosaccharides of glycoproteins are degraded from their non-reducing end by the sequential action of at least five different lysosomal enzyme activities. Deficiency of any of these activities results in the storage and excretion of the undegraded substrate(s). The lysosomal enzyme ß-mannosidase (EC 3.2.1.25) cleaves the N-linked mannosyl residue in the N-linked oligosaccharide side chains of glycoproteins and glycopeptides. Genetic disorders associated

with ß-mannosidase deficiency (ß-mannosidosis) have been described in Nubian goats (Jones and Dawson, 1981a; Healy et al., 1981; Jones et al., 1984; Pearce et al., 1987), Salers cattle (Abbitt et al., 1991; Jolly et al., 1991; Bryan et al., 1990) and humans (Cooper et al., 1986; Wenger et al., 1986; Dorland et al., 1988; Cooper et al., 1991; Kleijer et al., 1990). The disaccharide Manß1-4GlcNAc, the trisaccharide Manß1-4GlcNAcß1-4GlcNAc and other more complex oligosaccharides accumulate in the tissues and urine of affected goats and calves (Jones et al., 1984; Jones and Laine, 1981b; Matsuura et al., 1981; Matsuura et al., 1983; Jones et al., 1991; Gage et al., 1992). In humans the disaccharide Manß1-4GlcNAc is the major storage and excretion product (van Pelt et al., 1990; Tjoa et al., 1990). The accumulation of the trisaccharide in goats and calves is reportedly due to the absence of chitobiase in ruminants (Aronson and Kuranda, 1989), although the presence of lesser amounts of disaccharide suggests that ruminants may have some chitobiase activity (Hancock and Dawson, 1987).

Clinical and pathological abnormalities of ß-mannosidosis were first characterized in Nubian goats (Hartley and Blakemore, 1973; Jones and Dawson, 1981a; Jones et al., 1981b). Features expressed at birth include facial dysmorphism, doming of the skull, ocular abnormalities, intention tremor, ataxia, joint hyperextension, muscle atrophy, cytoplasmic vacuolation, nerve deafness, and central

nervous system hypomyelination (Jones et al., 1983; Lovell and Jones, 1983; Kumar et al., 1986; Jones, 1989). Without immediate and intensive care affected kids die within the first day or two of the neonatal period. The widespread vacuolation of cells (Jones et al., 1983) and severe neurological deficits associated with myelin abnormalities (Lovell and Jones, 1983; Jones, 1989) are the most prominent features of this disease. Nearly all cell types show some cytoplasmic vacuolation; thyroid follicular epithelium being most notably affected. Evidence of reduced thyroid function in affected goats has led to speculations that the reduced levels of thyroid hormone may be playing a role in the myelination defect (Boyer et al., 1990a). The myelination defect is characterized by a caudal-to-rostral pattern of decreasing myelin in the central nervous system. The absence of myelin is associated with a decrease in the number of oligodendrocytes and an increase in the number of bipolar cells (Boyer and Lovell, 1990b).

Clinical and pathological abnormalities of calves with ß-mannosidase deficiency are extremely similar to those observed in goats (Patterson et al., 1991; Abbitt et al., 1991; Jolly et al., 1991; Bryan et al., 1990; Jones et al., 1991; Lovell et al., 1991) with one striking exception. Unlike goats, affected calves responded to loud noises and had intact auditory brainstem evoked potentials (Render et al., 1992). A few other, more subtle, pathological differences have also

been described (Abbitt et al., 1991; Jolly et al., 1991; Bryan et al., 1990).

Clinical features of ß-mannosidase deficiency in humans differ significantly from those observed in ruminants. Clinical manifestations in humans can be mild and in the nine cases (among six families) reported thus far there is considerable heterogeneity (Kleijer et al., 1990; Cooper et al., 1991; Dorland et al., 1988; Wenger et al., 1986; Cooper et al., 1986). There is no clinical feature consistent across all cases. Patients typically present one or more of the following manifestations: mental retardation, hearing impairment or loss, aggressive behavior, angiokeratoma, seizures and recurrent infections of the respiratory tract and/or skin. Clinical heterogeneity is also present among siblings (Kleijer et al., 1990). An unusual patient with a combined deficiency of ß-mannosidase and heparin sulphate sulphamidase deficiency had coarse facial features and skeletal abnormalities which may have been due, at least in part, to the sulphatase deficiency (Wenger et al., 1986). The genetic or metabolic basis of the clinical heterogeneity among the human patients is currently unknown. The presence of heterogeneity among siblings suggests that it is not entirely due to allelic heterogeneity.

## ß-Mannosidase

ß-Mannosidase has been partially purified and incompletely characterized from several mammalian sources (Dawson, 1982; Noeske and Mersmann, 1983; Cavanagh et al., 1985; Bernard et al., 1986; Iwasaki et al., 1989; McCabe et al., 1990; McCabe and Dawson, 1991; Kyosaka et al., 1985; Frei et al., 1988; Sopher et al., 1992). Molecular weight estimates by gel filtration range from 80 to 120 kDa. The reported extent of purification as judged by the number of bands observed when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is low, except for guinea pig liver (Kyosaka et al., 1985) and goat kidney (Sopher et al., 1992).

Guinea pig ß-mannosidase was purified 8,000 fold with a recovery of 28% by Kyosaka and colleagues at Toho University in Japan (Kyosaka et al., 1985). The purified enzyme preparation reportedly contained a single peptide of 110 kDa as judged by Coomassie blue stained SDS-PAGE. As judged by gel filtration the purified enzyme had a molecular weight of approximately 120 kDa.

To characterize the enzyme further, McCabe and Dawson at the University of Chicago, generated polyclonal antiserum (anti-ß-mannosidase) using Kyosaka's purified enzyme preparation (McCabe and Dawson, 1991). In their hands Kyosaka's enzyme preparation contained a single peptide of 97 kDa as determined by Coomassie blue stained SDS-PAGE. The

reason for this size discrepancy is unclear. When used to probe a Western blot of purified ß-mannosidase (Kyosaka's enzyme preparation) the anti-ß-mannosidase serum reacted with a 97 kDa peptide and a 37 kDa peptide. The detection of a second peptide (37 kDa) in Kyosaka's enzyme preparation emphasizes the provisional nature of the identity of the 97 kDa peptide. When used to probe a Western blot of liver homogenate the anti-ß-mannosidase serum reacted with a 150 kDa peptide. Repeated freeze/thaw cycles prior to the addition of the SDS-PAGE sample buffer reportedly changed the size of the immunoreactive material to 120 and 20 kDa. More numerous freeze/thaw cycles or organic extraction of lipids prior to the addition of SDS-PAGE sample buffer reportedly gave rise to immunoreactive bands of 97, 37 and 20 kDa (McCabe and Dawson, 1991). The identity, relationship and functional significance of these associated peptides (37 and 20 kDa) relative to the 97 kDa peptide is unclear.

Goat ß-mannosidase has been purified 120,000 fold with a 26% yield from kidney tissue (Sopher et al., 1992). As estimated by gel filtration, the purified enzyme has a molecular weight of approximately 120 kDa. When analyzed by SDS-PAGE and visualized by silver staining the purified enzyme preparation contained three prominent peptides (80, 90 and 100 kDa). Two of these peptides (90 and 100 kDa) are both associated with ß-mannosidase activity in chromatographic fractions subsequent to immunoaffinity purification, react

with anti-ß-mannosidase monoclonal antibodies and produce similar electrophoretic peptide patterns when subjected to limited proteolysis. Given these observations, it appears that the 90 and 100 kDa peptides represent two related forms of caprine ß-mannosidase.

Genetic studies suggest that ß-mannosidase is encoded by a single structural gene. The segregation pattern of ß-mannosidosis in goats, cattle and humans is consistent with a simple autosomal recessive mutation. In all cases reported, affected individuals have a complete deficiency of tissue and plasma lysosomal ß-mannosidase and obligate carriers have intermediate levels. The human gene for ß-mannosidase has been tentatively mapped to human chromosome 4 using a panel of human/rodent hybrid cell lines containing varying numbers of human chromosomes (Fisher et al., 1987). A gene which influences the activity of ß-mannosidase in the mouse has been tentatively mapped to the distal end of mouse chromosome 3 (Lundin, 1987). These two findings are consistent with one another because the distal part of mouse chromosome 3 is syntenically related to the long arm of human chromosome 4.

Complementation studies using fibroblasts obtained from five human patients with ß-mannosidosis (including the patient with the combined ß-mannosidase/heparin sulphate sulphamidase deficiencies) and one affected goat demonstrated that none of these mutations complement one another (Hu et al., 1991). This suggests that all six of the ß-mannosidosis mutations

evaluated are allelic, including the combined deficiency. This further suggests that the combined deficiency arose either due to the rare occurrence of two independent mutations or due a large deletion which encompassed both the ß-mannosidase and the heparin sulphate sulphamidase loci. The chromosomal location of heparin sulphate sulphamidase is currently unknown.

## Overview

The long term specific aim of the three separate research projects presented here was to clone the ß-mannosidase gene. In the first project (chapter 1), the degree of linkage between a guinea pig cDNA (cloned with polyclonal anti-ß-mannosidase rabbit serum) and the caprine ß-mannosidosis disease locus was evaluated. In the second and third projects (chapter 2 and 3 respectively), anti-ß-mannosidase monoclonal antibodies were generated and used to purify and characterize the ß-mannosidase enzyme in caprine and bovine kidney tissues.

"Over the past five years, molecular studies have expanded our understanding of lysosomal enzymes and their genes both in the normal and in lysosomal storage diseases. The limiting step in this process has been the initial purification of the enzymes to homogeneity, in order to obtain antibodies as well as partial sequence for cloning cDNA" (Neufeld, 1991).

## REFERENCES

Abbitt, B., Jones, M.Z., Kasari, T.R., Storts, R.W., Templeton, J.W., Holland, P.S. and Castenson, P.E. ß-Mannosidosis in twelve Salers calves. J.Am.Vet.Med.Assoc. 198:109-113, 1991.

Aronson, N.N. and Kuranda, M.J. Lysosomal degradation of Asn-linked glycoproteins. FASEB J. 3:2615-2622, 1989.

Baranski, T.J., Faust, P.L. and Kornfeld, S. Generation of a lysosomal enzyme targeting signal in the secretory protein pepsinogen. Cell 63:281-291, 1990.

Baranski, T.J., Koelsch, G., Hartsuck, J.A. and Kornfeld, S. Mapping and molecular modeling of a recognition domain for lysosomal enzyme targeting. J.Biol.Chem. 266:23365-23372, 1991.

Bergmann, J.E. and Grabowski, G.A. Posttranslational processing of human lysosomal acid ß-glucosidase: A continuum of defects in Gaucher disease type 1 and type 2 fibroblasts. Am.J.Hum.Genet. 44:741-750, 1989.

Bernard, M., Sioud, M., Percheron, F. and Foglietti, M.J. ß-Mannosidase in human serum and urine. A comparative study. Internat.J.Biochem. 18:1065-1068, 1986.

Bielicki, J., Freeman, C., Clements, P.R. and Hopwood, J.J. Human liver iduronate-2-sulphatase. Purification, characterization and catalytic properties. Biochem.J. 271:75-86, 1990.

Boyer, P.J., Jones, M.Z., Nachreiner, R.F., Refsal, K.R., Common, R.S., Kelley, J. and Lovell, K.L. Caprine ß-mannosidosis: Abnormal thyroid structure and function in a lysosomal storage disease. Lab.Invest. 63:100-106, 1990a.

Boyer, P.J. and Lovell, K.L. In vitro characterization of oligodendrocytes in caprine ß-mannosidosis. Ann.NY Acad.Sci. 605:424-425, 1990b.

Bryan, L., Schmutz, S., Hodges, S.D. and Snyder, F.F. Bovine ß-mannosidase deficiency. Biochem.Biophys.Res.Comm. 173:491-495, 1990.

Cavanagh, K., Fisher, R.A., Legler, G., Herrchen, M., Jones, M.Z., Julich, E., Sewell-Alger, R.P., Sinnott, M.L. and Wilkinson, F.E. Goat liver ß-mannosidases: Molecular properties, inhibition and inactivation of the lysosomal and

nonlysosomal forms. Enzyme 34:75-82, 1985.

Clements, P.R., Brooks, D.A., McCourt, P.A. and Hopwood, J.J. Immunopurification and characterization of human α-L-iduronidase with the use of monoclonal antibodies. Biochem.J. 259:199-208, 1989.

Cooper, A., Sardharwalla, I.B. and Roberts, M.M. Human ß-mannosidase deficiency. N.Engl.J.Med. 315:1231, 1986.

Cooper, A., Wraith, J.E., Savage, W.J., Thornley, M. and Noronha, M.J. ß-Mannosidase deficiency in a female infant with epileptic encephalopathy. J.Inherited Metab.Dis. 14:18-22, 1991.

Dahms, N., Lobel, P. and Kornfeld, S. Mannose 6-phosphate receptors and lysosomal enzyme targeting. J.Biol.Chem. 264:12115-12118, 1989.

Dawson, G. Evidence for two distinct forms of mammalian ß-mannosidase. J.Biol.Chem. 257:3369-3371, 1982.

d'Azzo, A., Hoogeveen, A., Reuser, A.J.J., Robinson, D. and Galjaard, H. Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. Proc.Natl.Acad.Sci.USA 79:4535-4539, 1982.

Deval, C., Bechet, D., Obled, A. and Ferrara, M. Purification and properties of different isoforms of bovine cathepsin B. Biochem.Cell.Biol. 68:822-826, 1990.

DiCioccio, R.A. and Mahoney, C.M. Effect of glycosylation inhibitors and acidotropic amines on the synthesis, processing, and intracellular-extracellular distribution of α-L-fucosidase in B-lymphoblastoid cells. Carbohydr.Res. 197:217-226, 1990.

Dorland, L., Duran, M., Hoefnagels, F.E.T., Breg, J.N., Fabery de Jonge, H., van Eghen-Cransberg, K., van Sprang, F.J. and van Diggelen, O.P. ß-Mannosidosis in two brothers with hearing loss. J.Inherited Metab.Dis. 11:255-258, 1988.

Erickson, A.H. Biosynthesis of lysosomal endopeptidases. J.Cell.Biochem. 40:31-41, 1989.

Fisher, R.A., Povey, S., Cavanagh, K., Dupuis, M. and Jones, M.Z. Studies to determine the chromosome assignment of human lysosomal ß-mannosidase (MANBA). Am.J.Hum.Genet. 41:A165, 1987.

Frei, J.I., Cavanagh, K., Fisher, R.A., Hausinger, R.P., Dupis, M., Rathke, E.J.S. and Jones, M.Z. Partial purification

of goat kidney ß-mannosidase. Biochem.J. 249:871-875, 1988.

Frisch, A. and Neufeld, E.F. Limited proteolysis of the ß-hexosaminidase precursor in a cell-free system. J.Biol.Chem. 256:8242-8246, 1981.

Gage, D.A., Rathke, E.J.S., Costello, C.E. and Jones, M.Z. Determination of sequence and linkage of tissue oligosaccharides in caprine ß-mannosidosis by FAB-CAD-MS/MS. Glycoconjugate J. (in press), 1992.

Galjart, N.J., Gilleman, N., Harris, A., van der Horst, G.T.J., Verheijen, F.W., Galjaard, H. and d'Azzo, A. Expression of cDNA encoding the human "protective protein" associated with lysosomal ß-galactosidase and neuraminidase: Homology to yeast proteases. Cell 54:755-764, 1988.

Gottschalk, S., Waheed, A. and von Figura, K. Targeting of lysosomal acid phosphatase with altered carbohydrate. Biol.Chem.Hoppe-Seyler 370:75-80, 1989.

Hancock, L.W. and Dawson, G. Evidence for two catabolic endoglycosidase activites in ß-mannosidase-deficient goat fibroblasts. Biochim.Biophys.Acta 928:13-21, 1987.

Hartley, W.J. and Blakemore, W.F. Neurovisceral storage and dysmyelinogenesis in neonatal goats. ActaNeuropathol. 25:325-333, 1973.

Hasilik, A. and Neufeld, E.F. Biosynthesis of lysosomal enzymes in fibroblasts. J.Biol.Chem. 255:4937-4945, 1980.

Healy, P.J., Seaman, J.T., Gardner, I.A. and Sewell, C.A. ß-Mannosidase deficiency in anglo nubian goats. Aust.Vet.J. 57:504-507, 1981.

Hoefsloot, L.H., Willemsen, R., Kroos, M.A., Hoogeveen-Westerveld, M., Hermans, M.M.P., van der Ploeg, A.T., Oostra, B.A. and Reuser, A.J.J. Expression and routeing of human lysosomal α-glucosidase in transiently transfected mammalian cells. Biochem.J. 272:485-492, 1990.

Hoeksema, H.L., van Diggelen, O.P. and Galjaard, H. Intergenic complementation after fusion of fibroblasts from different patients with ß-galactosidase deficiency. Biochim.Biophys.Acta 566:72-79, 1979.

Holtzman, E. Extensive release. Excessive storage. In: Lysosomes, New York: Plenum Press, 1989a, pp. 319-359.

Holtzman, E. Genesis. In: Lysosomes, New York: Plenum Press,

1989b, pp. 363-416.

Hu, P., Wenger, D.A., van Diggelen, O.P. and Kleijer, W.J. Complementation studies in human and caprine ß-mannosidosis. J.Inherited Metab.Dis. 14:13-17, 1991.

Iwasaki, Y., Tsuji, A., Omura, K. and Suzuki, Y. Purification and characterization of ß-mannosidase from human placenta. J.Biochem. 106:331-335, 1989.

Jolly, R.D., Thompson, K.G., Bayliss, S.L., Vidler, B.M., Orr, M.B. and Healy, P.J. ß-Mannosidosis in a Salers calf: A new storage disease of cattle. J.New Zealand Vet.Assn. (in press), 1991.

Jones, M.Z. and Dawson, G. Caprine ß-mannosidosis: Inherited deficiency of ß-mannosidase. J.Biol.Chem. 256:5185-5188, 1981a.

Jones, M.Z. and Laine, R.A. Caprine oligosaccharide storage disease: Accumulation of ß-mannosyl (1-4) ß-N-acetylglucosaminyl (1-4) ß-N-acetylglucosamine in brain. J.Biol.Chem. 256:5181-5184, 1981b.

Jones, M.Z., Cunningham, J.G., Dade, A.W., Alessi, D.W., Mostosky, U.V., Vorro, J.R., Benitez, J.T. and Lovell, K.L.

Caprine ß-mannosidosis: Clinical and pathological features. J.Neuropath.Exp.Neurol. 42:268-285, 1983.

Jones, M.Z., Rathke, E.J.S., Cavanagh, K. and Hancock, L.W. ß-Mannosidosis: Prenatal biochemical and morphological characteristics. J.Inherited Metab.Dis. 7:80-85, 1984.

Jones, M.Z. ß-Mannosidosis. Comp.Pathol.Bull. 21:2-4, 1989.

Jones, M.Z., Rathke, E.J.S., Gage, D.A., Costello, C.E., Murakami, K., Ohta, M. and Matsura, F. Oligosaccharides accumulated in the bovine ß-mannosidosis kidney. J.Inherited Metab.Dis. (in press), 1991.

Kleijer, W.J., Hu, P., Thoomes, R., Boer, M., Huijmans, J.G.M., Blom, W., van Diggelen, O.P., Seemanova, E. and Macek, M. ß-Mannosidase deficiency: Heterogeneous manifestations in the first female patient and her brother. J.Inherited Metab.Dis. 13:867-872, 1990.

Kornfeld, S. and Mellman, I. The biogenesis of lysosomes. Annu.Rev.Cell Biol. 5:483-525, 1989.

Kornfeld, S. Lysosomal enzyme targeting. Biochem.Soc.Trans. 18:367-374, 1990.

Kumar, K., Jones, M.Z., Cunningham, J.G., Kelley, J.A. and Lovell, K.L. Caprine ß-mannosidosis: Phenotypic features. Vet.Rec. 118:325-327, 1986.

Kyosaka, S., Murata, S., Nakamura, F. and Tanaka, M. Purification and kinetic properties of guinea pig liver ß-mannosidase. Chem.Pharm.Bull. 33:256-263, 1985.

Lovell, K.L. and Jones, M.Z. Distribution of central nervous system lesions in ß-mannosidosis. ActaNeuropathol. 62:121-126, 1983.

Lovell, K.L., Jones, M.Z., Patterson, J., Abbitt, B. and Castenson, P. Thyroid structure and function in bovine ß-mannosidosis. J.Inherited Metab.Dis. 14:228-230, 1991.

Lundin, L.G. A gene (Bmn) controlling ß-mannosidase activity in the mouse is located in the distal part of chromosome 3. Biochem.Genet. 25:603-610, 1987.

Mahuran, D.J., Neote, K., Klavins, M.H., Leung, A. and Gravel, R.A. Proteolytic processing of pro-α and pro-ß precursors from human ß-hexosaminidase. Generation of the mature α and ß subunits. J.Biol.Chem. 263:4612-4618, 1988.

Matsuura, F., Laine, R.A. and Jones, M.Z. Oligosaccharides accumulated in the kidney of a goat with ß-mannosidosis: Mass spectrometry of intact permethylated derivatives. Arch.Biochem.Biophys. 211:485-493, 1981.

Matsuura, F., Jones, M.Z. and Frazier, S.E. Structural analysis of the major caprine ß-mannosidosis urinary oligosaccharides. Biochim.Biophys.Acta 759:67-73, 1983.

McCabe, N.R., Biliter, W. and Dawson, G. Preferential inhibition of lysosomal ß-mannosidase by sucrose. Enzyme 43:137-145, 1990.

McCabe, N.R. and Dawson, G. A study of the heterogenous structure of guinea pig lysosomal ß-mannosidase using a polyclonal antibody. Biochim.Biophys.Acta 1077:133-140, 1991.

Myerowitz, R. and Neufeld, E.F. Maturation of α-iduronidase in cultured human fibroblasts. J.Biol.Chem. 256:3044-3048, 1981.

Neufeld, E.F. Lysosomal storage diseases. Annu.Rev.Biochem. 60:257-280, 1991.

Noeske, C. and Mersmann, G. ß-D-Mannosidase from human placenta: Properties and partial purification. Hoppe-Seyler'sZ.Physiol.Chem. 364:1645-1651, 1983.

Patt
Neu
J.Ne

Pear
Kieh
of
243:

Poti
Stru
carb
1990

Quor
Neuf
lysc
fibr

Rend
otic
pres

Rijn
Stro
asso

Patterson, J.S., Jones, M.Z., Lovell, K.L. and Abbitt, B. Neuropathology of bovine ß-mannosidosis. J.Neuropathol.Exp.Neurol 50:538-545, 1991.

Pearce, R.D., Callahan, J.W., Little, P.B., Armstrong, D.T., Kiehm, D. and Clarke, J.T.R. Properties and prenatal ontogeny of ß-mannosidase in selected goat tissues. Biochem.J. 243:603-609, 1987.

Potier, M., Michaud, L., Tranchemontagne, J. and Thauvette, L. Structure of the lysosomal neuraminidase-ß-galactosidase-carboxypeptidase multienzymic complex. Biochem.J. 267:197-202, 1990.

Quon, D.V.K., Proia, R.L., Fowler, A.V., Bleibaum, J. and Neufeld, E.F. Proteolytic processing of the ß-subunit of the lysosomal enzyme, ß-hexosaminidase, in normal human fibroblasts. J.Biol.Chem. 264:3380-3384, 1989.

Render, J.A., Lovell, K.L. and Keller, C.B. The ocular and otic pathology of bovine ß-mannosidosis. J.Vet.Diag.Invest (in press), 1992.

Rijnboutt, S., Aerts, H.M.F.G., Geuze, H.J., Tager, J.M. and Strous, G.J. Mannose 6-phosphate-independent membrane association of cathepsin D, glucocerebrosidase, and

sphingolipid-activating protein in hepG2 cells. J.Biol.Chem. 266:4862-4868, 1991.

Skudlarek, M., Novak, E. and Swank, R.T. Lysosomes in biology and pathology, Amsterdam: Elsevier, 1984.

Sopher, B.S., Traviss, C.E., Cavanagh, K.T., Jones, M.Z. and Friderici, K.H. Purification and characterization of goat lysosomal ß-mannosidase using monoclonal and polyclonal antibodies. J.Biol.Chem. (in press), 1992.

Taylor, J.A., Gibson, G.J., Brooks, D.A. and Hopwood, J.J. Human N-acetylgalactosamine-4-sulphatase biosynthesis and maturation in normal, Maroteaux-Lamy and multiple-sulphatase-deficient fibroblasts. Biochem.J. 268(2):379-386, 1990.

Tjoa, S., Wenger, D.A. and Fennessey, P.V. Quantitative analysis of disaccharides in the urine of ß-mannosidosis patients. J.Inherited Metab.Dis. 13:187-194, 1990.

Tsuji, A., Omura, K. and Suzuki, Y. I-cell disease: Evidence for a mannose 6-phosphate independent pathway for translocation of lysosomal enzymes in lymphoblastoid cells. Clin.Chim.Acta 176:115-122, 1988.

van Diggelen, O.P., Hoogeveen, A.T., Smith, P.J., Reuser, A.J.J. and Galjaard, H. Enhanced proteolytic degradation of normal ß-galactosidase in the lysosomal storage disease with combined ß-galactosidase and neurominidase deficiency. Biochim.Biophys.Acta 703:69-76, 1982.

van Pelt, J., Hokke, C.H., Dorland, L., Duran, M., Kamerling, J.P. and Vliegenthart, J.F.G. Accumulation of mannosyl-ß(1-4)-N-acetylglucosamine in fibroblasts and leukocytes of patients with a deficiency of ß-mannosidase. Clin.Chim.Acta 187:55-60, 1990.

von Figura, K. and Hasilik, A. Lysosomal enzymes and their receptors. Ann.Rev.Biochem. 55:167-193, 1986.

Waheed, A., Gottschalk, S., Hille, A., Krentler, C., Pohlmann, R., Braulke, T., Hauser, H., Gevze, H. and von Figura, K. Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells. EMBOJ. 7:2341-2358, 1988.

Wenger, D.A., Sujansky, E., Fennessey, P.V. and Thompson, J.N. Human ß-mannosidase deficiency. N.Engl.J.Med. 315:1201-1205, 1986.

**Characterization of a Guinea Pig cDNA Clone: Human Chromosomal Localization and Linkage Analysis Between the Cloned Gene and the Caprine ß-Mannosidosis Disease Gene**

## ABSTRACT

Chromosome mapping and pedigree segregation analysis were used to evaluate the likelihood that a guinea pig cDNA of interest, obtained from Glyn Dawson at the University of Chicago, encodes ß-mannosidase. Molecular linkage analysis of a goat pedigree shows that the guinea pig cDNA sequence (which mapped to human chromosome 4) does not consistently segregate with the caprine ß-mannosidosis disease gene. These findings demonstrate that the cloned guinea pig gene does not correspond to the caprine ß-mannosidosis disease gene and strongly suggest that the gene does not encode ß-mannosidase.

# INTRODUCTION

The guinea pig cDNA evaluated in this study was obtained from Glyn Dawson (University of Chicago, Department of Pediatrics). McCabe and Dawson isolated the clone from a guinea pig liver cDNA library using rabbit antiserum which detected one band (150 kDa) when used to probe a Western blot of guinea pig liver crude homogenate (McCabe and Dawson, 1991). Dawson and co-workers sequenced the clone (320 bp) and found it to contain one open reading frame which encoded a peptide that did not share sequence similarity (> 40% identity) with sequences in the GCG database (personal communication).

In this study chromosome mapping and linkage studies were carried out to evaluate the likelihood that this guinea pig cDNA encodes ß-mannosidase. The autosomal recessive gene for caprine ß-mannosidosis has been maintained for four generations in a small colony of Nubian goats housed on the Michigan State Campus. The genealogy of the family pedigree is known and the genotype with respect to ß-mannosidosis has been documented. Prior to these studies, ß-mannosidase was tentatively mapped to human chromosome 4 (Fisher et al., 1987).

## EXPERIMENTAL PROCEDURES

Materials-Proteinase K was purchased from Sigma, St. Louis, MO. Hybond-N and $dCTP^{32}$ were purchased from Amersham, Arlington Heights, IL. A Random Primed DNA labeling kit was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Pedigree-The clinical presentation of caprine ß-mannosidosis has been reported elsewhere (Jones et al., 1983; Kumar, et al., 1986). The autosomal recessive ß-mannosidosis disease mutation has been maintained for four generations in a colony of goats housed on the Michigan State University campus, East Lansing, MI.

DNA Isolation-DNA was isolated from leukocytes or spleen tissue. Leukocyte DNA was isolated from 50 ml of whole blood that had been collected in sodium heparin. Four volumes of lysis buffer (155 mM $NH_4Cl$, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4, 4 °C) were added to the heparinized blood sample which was incubated on ice for 10 minutes. After centrifugation (500 x g, 5 min), the white cell pellet was resuspended in 50 ml of lysis buffer, incubated on ice for 10 minutes, diluted with 6 volumes of 0.9% NaCl, and centrifuged (500 x g, 5 min). The cell pellet was subjected to a second cycle of resuspension and centrifugation. The pellet was resuspended in 5 ml of DNA buffer (10 mM Tris-Cl (pH 7.5), 25 mM EDTA, and 100 mM NaCl) and the cells lysed by addition of sodium dodecyl sulphate (SDS) and Proteinase K (a final concentration of 0.2% and 0.4

mg/ml respectively). The sample was incubated at 37 °C overnight and stored at 4 °C prior to phenol extraction.

Spleen DNA was isolated from spleen samples stored at -20 °C. Spleen tissue (0.5 g) was homogenized in DNA buffer using a glass vessel with a motor-driven teflon pestle. The homogenate was filtered through a sterile gauze pad. The filtered homogenate was diluted with DNA buffer to 50 ml and SDS and Proteinase K were added to a final concentration of 0.2% and 0.4 mg/ml respectively. The sample was incubated at 37 °C overnight and stored at 4 °C prior to phenol extraction.

Extraction, ethanol precipitation, and dialysis of the DNA samples were carried out using standard procedures (Sambrook et al., 1989).

Enzyme Digestion and Southern Transfer-Restriction enzyme digestions were carried out according to manufacturer's specifications; enzyme digestions included 4 mM spermidine. DNA fragments were separated by 0.8% agarose-gel electrophoresis. Following electrophoresis, the gels were processed and transferred to Hybond-N as described (Sambrook et al., 1989).

Somatic Cell Hybrids-DNA from human-rodent hybrid cells, containing known complements of human chromosomes, was analyzed by Southern analysis. The DNA was obtained from Sue Povey at The Galton Laboratory, University College, London, England.

Probes and Hybridization-The entire 320 bp cDNA EcoRI insert (referred to as GPC320) was used to probe the panel of human-rodent DNA. To simplify the TaqI restriction fragment pattern, a 180 bp EcoRI-PvuII sub-fragment (referred to as GPC180) was used when probing goat DNA. The probes were denatured and labeled, according to manufacturer's instructions, with $\alpha$-[$^{32}$P]-dCTP to a high specific activity (typically 5 x $10^8$-5 x $10^9$ cpm/ug DNA) using the random priming method. Pre-hybridization was carried out for 2 hrs at 65 °C in a solution containing 5 x SSPE, 5 x Denhardt's, 0.5% SDS, and sonicated non-homologous DNA (0.05 mg/ml). The denatured probe was added and hybridization was carried out overnight at 65 °C. After hybridization the filters were washed at 65 °C, in 3 x SSC and 0.1% SDS for 20 min and subsequently at increasing stringencies (1-0.1 x SSC with 0.1% SDS), depending on the filter. The washed filter was placed against X-ray film (Kodak X-OMat AR) with intensifying screens for 1-7 days at -70 °C for autoradiography.

## RESULTS

Human Chromosomal Localization of GPC320-To determine if the human gene, homologous to GPC320, is on human chromosome 4, a somatic cell hybrid panel was analyzed. When GPC320 was used to probe a Southern blot of a set of human-rodent DNA,

the hybridization pattern was consistent with the presence/absence of human chromosome 4, except for cell line FST 9/7 (Fig. 1). Comparison of the hybridization pattern of the GPC320 sequence with human chromosomes shows the highest concordance fraction was for chromosome 4 (93%), followed by chromosome 22 (75%), chromosome 9 (70%) and chromosome 12 (69%) (Table 1). These observation suggest that the cloned guinea pig gene is localized on human chromosome 4.

Linkage Analysis Between the GPC180 Sequence and the Caprine ß-Mannosidosis Disease Locus-Southern analysis of DNA (digested with TaqI and probed with GPC180) from selected members of the "ß-mannosidosis" goat colony revealed three different alleles of the gene cloned by McCabe and Dawson. To evaluate the segregation pattern of these three alleles (in the goat colony), DNA from a family of 18 goats was digested with TaqI, subjected to Southern analysis and probed with GPC180 (Fig. 2).

## DISCUSSION

Analysis of the hybridization pattern of GPC320 to a human-rodent somatic cell hybrid panel indicates that the human gene, homologous to the cloned guinea pig gene, is on human chromosome 4. This is consistent with the tentative chromosomal location of human ß-mannosidase.

If GPC180 encodes the ß-mannosidosis disease gene, then only one allele of the homologous goat gene (of the three revealed by RFLP analysis) should segregate with caprine ß-mannosidosis in our goat colony. For example, consider the segregation of the disease gene from goat 24 to goat 213 in Fig. 3. Goats 142 and 24 produced two offspring (143 and 144). These goats (143 and 144) produced an affected offspring (213) and are therefore obligate carriers. Because goat 142 has normal levels of serum ß-mannosidase and because she is unrelated to the other goats in the colony, one can have extreme confidence that she is homozygous normal. That implies that goats 143 and 144 received the ß-mannosidosis disease allele from their father (goat 24, an obligate carrier). Because goats 143 and 144 received the S allele of the cloned gene from their father (goat 24) one can deduce that the S allele of the cloned gene should represent the disease allele. Therefore, if recombination is not occurring between the disease mutation and the S allele of the cloned gene, one would expect that goat 213 would have received an S alleles from both of his parents. However, goat 213 actually received two L alleles of the cloned gene which implies that recombination has occurred between the cloned gene and the disease mutation in both his father and his mother (goats 144 and 143 respectively). Given the frequency of recombination between the GPC180 sequence and the disease mutation (see discordant segregation in Fig. 3) it is very clear that the

GPC180 sequence cloned by McCabe and Dawson does not correspond to the ß-mannosidosis disease gene. To maintain that the cloned guinea pig gene is ß-mannosidase, one would have to argue that the function of the ß-mannosidase enzyme is dependent on at least two non-allelic peptides. Characterization of purified ß-mannosidase from goat kidney (Sopher et al., 1992) and guinea pig liver (Kyosaka et al., 1985) suggests that the enzyme is not composed of multiple gene products. Consequently, one can be quite confident that the cDNA cloned by McCabe and Dawson does not encode ß-mannosidase.

Acknowledgments-I would like to thank Dr. Karen Friderici for the helpful consultations and the technical guidance.

## REFERENCES

Fisher, R.A., Povey, S., Cavanagh, K., Dupuis, M. and Jones, M.Z. Studies to determine the chromosome assignment of human lysosomal ß-mannosidase (MANBA). Am.J.Hum.Genet. 41:A165, 1987.

Jones, M.Z., Cunningham, J.G., Dade, A.W., Alessi, D.W., Mostosky, U.V., Vorro, J.R., Benitez, J.T. and Lovell, K.L. Caprine ß-mannosidosis: Clinical and pathological features. J.Neuropath.Exp.Neurol. 42:268-285, 1983.

Kumar, K., Jones, M.Z., Cunningham, J.G., Kelley, J.A. and Lovell, K.L. Caprine ß-mannosidosis: Phenotypic features. Vet.Rec. 118:325-327, 1986.

Kyosaka, S., Murata, S., Nakamura, F. and Tanaka, M. Purification and kinetic properties of guinea pig liver ß-mannosidase. Chem.Pharm.Bull. 33:256-263, 1985.

McCabe, N.R. and Dawson, G. A study of the heterogenous structure of guinea pig lysosomal ß-mannosidase using a polyclonal antibody. Biochim.Biophys.Acta 1077:133-140, 1991.

Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular cloning: A laboratory manual, New York: Cold Spring Harbor

Laboratory Press, 1989.

Sopher, B.S., Traviss, C.E., Cavanagh, K.T., Jones, M.Z. and Friderici, K.H. Purification and characterization of goat lysosomal ß-mannosidase using monoclonal and polyclonal antibodies. J.Biol.Chem. (in press), 1992.

Table I

Correlation of GPC320 Gene Sequences with Human Chromosomes in Somatic Cell Hybrids

| | Human Chromosomes[a] | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | GPC320 |
| Hybrids: | | | | | | | | | | | | | | | | | | | | | | | | |
| Human-mouse: | | | | | | | | | | | | | | | | | | | | | | | | |
| CTP 34B4 | + | + | + | - | + | + | + | + | - | - | - | + | - | + | - | + | + | + | - | - | - | - | + | - |
| MOG 2E5 | + | - | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | - | N | + | + | + | + |
| CRAB 2 | - | + | + | + | + | + | - | N | N | + | - | + | + | N | N | + | N | + | + | - | N | N | + | + |
| CRAB 7 | - | - | + | - | + | - | + | N | + | + | + | N | + | + | N | - | + | - | + | N | N | N | + | - |
| Human-rat: | | | | | | | | | | | | | | | | | | | | | | | | |
| FHA7 | - | - | + | + | + | + | + | + | N | N | - | + | N | N | + | + | + | + | N | + | - | N | + | + |
| FST 9/10 | - | - | + | + | - | + | - | + | - | + | - | + | + | + | + | - | - | + | - | + | - | + | + | + |
| FST 9/2 | - | - | + | - | - | + | N | + | - | + | - | + | - | + | + | - | - | + | - | - | - | N | + | - |
| FST 9/5 | - | - | + | + | - | + | N | + | N | + | - | + | - | + | + | - | - | + | - | + | - | N | + | + |
| FST 9/7 | - | - | + | + | - | + | N | + | N | + | - | + | + | + | + | - | - | + | - | + | - | N | + | - |
| SIF 15P5 | - | + | - | - | - | + | + | - | - | + | - | - | - | + | + | - | - | - | - | + | - | - | + | - |
| SIF 4A24E1 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | + | - | + | + |
| G10 E8 | - | + | - | - | + | - | - | + | - | - | - | - | - | - | + | - | - | + | - | - | + | - | + | - |
| Human-hamster: | | | | | | | | | | | | | | | | | | | | | | | | |
| TWIN 19F9 | + | N | + | + | - | + | - | + | + | - | - | + | N | + | - | - | + | + | - | + | + | + | - | + |
| TWIN 16 | + | - | N | - | - | - | N | + | - | - | + | - | N | + | N | + | + | N | - | + | - | + | + | - |
| % Discordancy:[b] | 50 | 46 | 38 | 7 | 50 | 36 | 70 | 50 | 30 | 46 | 64 | 31 | 36 | 58 | 55 | 43 | 38 | 38 | 46 | 42 | 33 | 25 | 57 | |

[a] + = Presence of the human chromosome; - = absence of the human chromososme; N = not known.
[b] Discordancy indicates the presence of the gene sequence but the absence of a specific chromosome, or the reverse.
% Discordancy = the percentage of discordant hybrids of the total hybrids.

Fig. 1. **Southern blot analysis of EcoRI-digested DNA from parental and human-rodent hybrid cells, using the GPC320 cDNA as a probe.** Hybrids containing human chromosome 4: MOG 2E5, CRAB 2, FHA7, FST 9/10, FST 9/5, FST 9/7 and TWIN 19F9.

Fig. 1

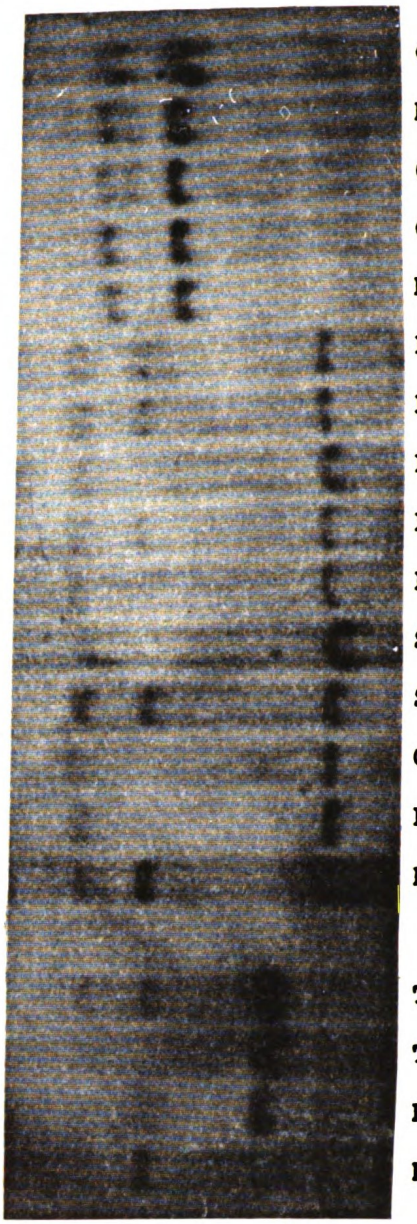

CTP 34B4
MOG 2E5
CRAB 2
CRAB 7
Mouse
FHA7
FST 9/10
FST 9/2
FST 9/5
FST 9/7
SIF 15P5
SIF 4A24E1
G10 E8
Rat
Human
TWIN 19F9
TWIN 16
Hamster
Human
1 kb

Fig. 2. **Southern blot analysis of TaqI-digested DNA from a family of goats, using the GPC180 cDNA as a probe.** Goat identification numbers are indicated above the blot. The genealogy of the family is given in Fig. 3. The location of molecular-weight markers (in kb) are indicated. Alleles of the GPC180 sequence are marked L (large), M (medium), and S (small).

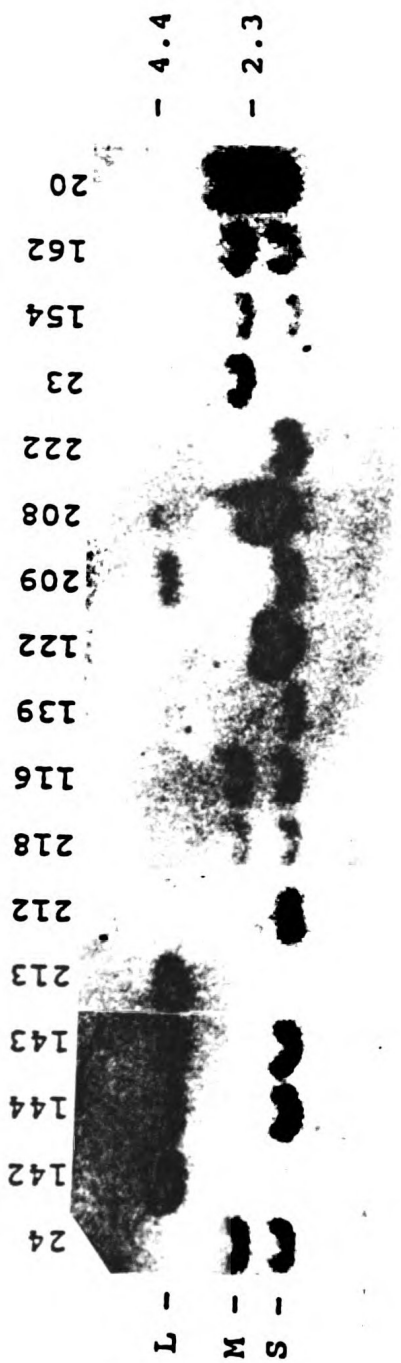
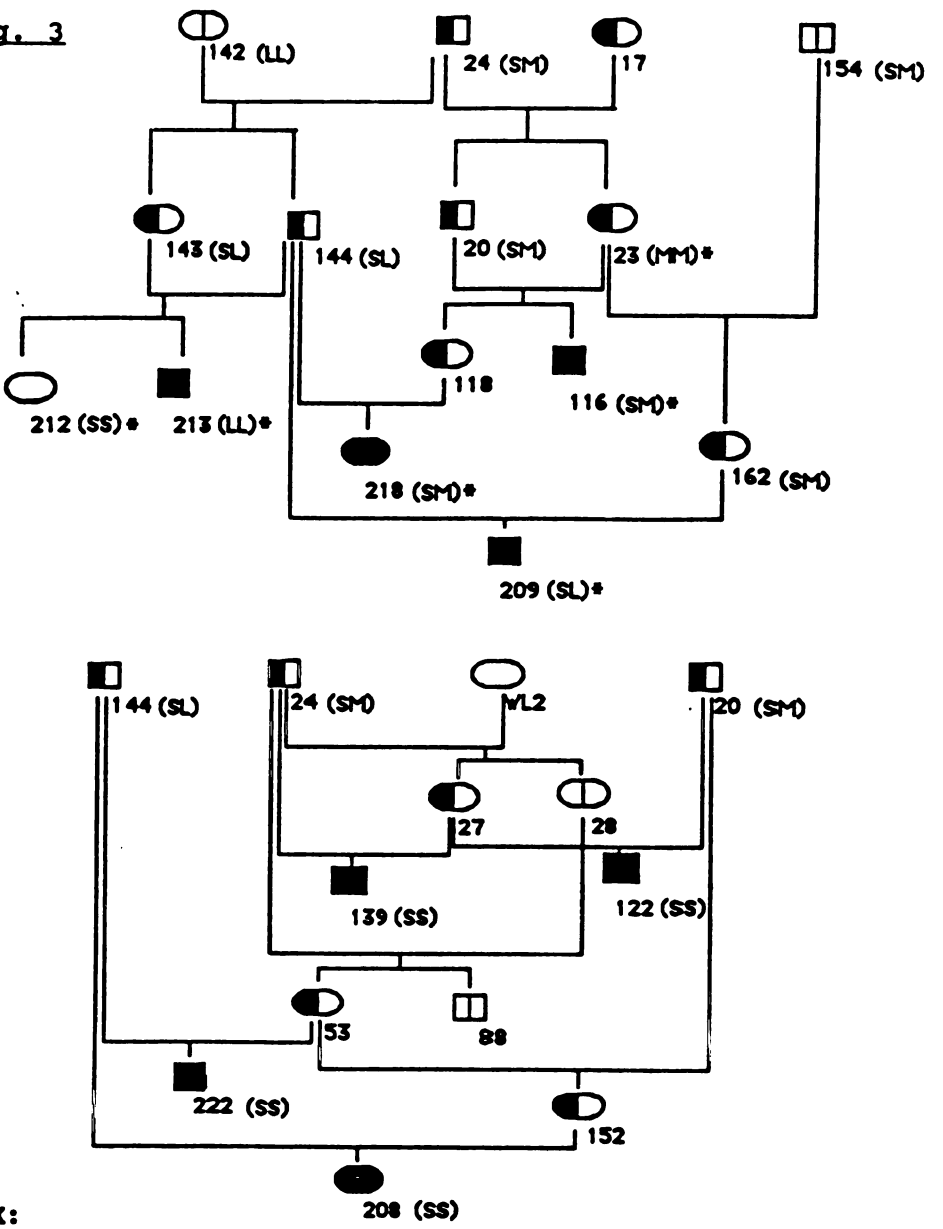

- 4.4
- 2.3
20
162
154
23
222
208
209
122
139
116
218
212
213
143
144
142
24
L -
M -
S -

Fig. 2

**Fig. 3. A caprine ß-mannosidosis disease pedigree.** The segregation of the disease gene and the GPC180 sequence are outlined.

Fig. 3




INDEX:

- Male (serum ß-mannosidase level - not tested)
- Male (serum ß-mannosidase level - normal)
- Male (affected)
- Male (obligate carrier)
- Female

L = Large TaqI restriction fragment (Fig. 2)
M = Medium TaqI restriction fragment (Fig. 2)
S = Small TaqI restriction fragment (Fig. 2)
* = Discordant segregation of the guinea pig gene and the ß-mannosidosis disease gene.

# Purification and Characterization of Goat Lysosomal ß-Mannosidase Using Monoclonal and Polyclonal Antibodies

fr

fo

Wh

el

pu

pe

mo

pe

De

pe

la

wi

th

re

re

## ABSTRACT

Goat ß-mannosidase was purified 120,000 fold in 26% yield from kidney using Concanavalin A-Sepharose chromatography followed by immunoaffinity and cation-exchange chromatography. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining, the purified enzyme preparation consists of 90 and 100 kDa peptides. Both these peptides react with anti-ß-mannosidase monoclonal antibodies and produce similar electrophoretic peptide patterns when subjected to limited proteolysis. Deglycosylation reduces the size of the 90 and 100 kDa peptides to 86 and 91 kDa respectively. Goat kidney tissues lacking ß-mannosidase activity, acquired from animals affected with ß-mannosidosis, do not contain detectable quantities of the 90 and 100 kDa peptides as judged by monoclonal antibody reactivity. We postulate that the 90 and 100 kDa peptides represent two related forms of ß-mannosidase.

# INTRODUCTION

N-linked oligosaccharides of glycoproteins are degraded from their non-reducing end by the sequential action of at least five different lysosomal enzyme activities. Deficiencies of any of these activities results in an accumulation of the incompletely catabolized oligosaccharide chains leading to widespread cellular vacuolation and a broad spectrum of clinical features, ranging from death in infancy to mild disorders of adulthood. The lysosomal enzyme ß-mannosidase (EC 3.2.1.25) cleaves the ß-linked mannosyl residue in the N-linked oligosaccharide side chains of glycoproteins. Genetic disorders associated with ß-mannosidase deficiency have been described in Nubian goats (Jones and Dawson, 1981a; Healy et al., 1981; Jones et al., 1984; Pearce et al., 1987), Salers cattle (Abbitt et al., 1991; Jolly et al., 1991; Bryan et al., 1990) and humans (Wenger et al., 1986; Dorland et al., 1988; Cooper et al., 1991; Kleijer et al., 1990). Goats and calves with ß-mannosidase deficiency are characterized by wide-spread vacuolation of cells (Jolly et al., 1991; Jones et al., 1984) and severe neurological deficits associated with myelin abnormalities (Jones et al., 1983; Lovell and Boyer, 1987; Boyer et al., 1990). The disaccharide Manß1-4GlcNAc, the trisaccharide Manß1-4GlcNAcß1-4GlcNAc and other more complex oligosaccharides accumulate in the tissues and urine of these animals (Jones et al., 1984; Jones and Laine, 1981b; Matsuura

et al., 1981; Matsuura et al., 1983; Jones et al., 1991). Without intervention goats and calves affected by ß-mannosidosis will die within the first day or two of life.

Clinical features of ß-mannosidase deficiency in humans differ significantly from those observed in ruminants. Clinical manifestations in humans can be mild and in the human cases reported thus far there is considerable clinical heterogeneity (Kleijer et al., 1990). In general, affected individuals are characterized by mental retardation, deafness, and in older patients angiokeratoma. In humans the disaccharide is the major storage product (van Pelt et al., 1990; Tjoa et al., 1990).

ß-mannosidase has been partially purified and incompletely characterized from several mammalian sources (Dawson, 1982; Noeske and Mersmann, 1983; Cavanagh et al., 1985; Kyosaka et al., 1985; Bernard et al., 1986; Iwasaki et al., 1989; McCabe et al., 1990; McCabe and Dawson, 1991; Frei et al., 1988). Molecular weight estimates by gel filtration range from 80 to 120 kDa. The reported extent of purification as judged by the number of bands observed when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is low, except for guinea pig liver where a major peptide of 110 kDa was demonstrated (Kyosaka et al., 1985).

I have undertaken the purification and characterization of lysosomal ß-mannosidase from normal goat kidney to make possible the biochemical, immunological and structural

ch
th
ma
ru
th
ß-
co
a
pu
an
the
198

Gro
mat
hou
wer
A-S
man
pro
were
A-ag

characterization of the enzyme, its corresponding gene(s), and the mutations which give rise to ß-mannosidosis. These studies may lead to insights regarding the myelin deficits in ruminants, the clinical heterogeneity in humans and possibly the biochemical basis of a combined deficiency of ß-mannosidase and heparin sulfamidase described by Wenger and colleagues (Wenger et al., 1986). This is the first report of a ß-mannosidase purification procedure that yielded highly purified peptides which are modified or absent in tissue from animals affected with ß-mannosidosis. This work builds upon the preliminary findings of Frei and co-workers (Frei et al., 1988).

## EXPERIMENTAL PROCEDURES

Materials-Kidneys from mature goats were obtained from M Gross Abattoir in Toronto, Canada. ß-Mannosidosis affected and matched control tissues were acquired from our goat colony housed on the Michigan State University campus. All tissues were stored at -20 °C or -80 °C until needed. Concanavalin A-Sepharose 4B (Con A-Sepharose), 4-methylumbelliferyl ß-D-mannopyranoside, methyl-α-glucoside, Staphylococcus aureus V8 protease, incomplete Freund's adjuvant and Coomassie blue dye were from Sigma, St. Louis, MO. Leupeptin, pepstatin, protein A-agarose, N-Glycosidase F and Endoglycosidase H were from

Boe
bea
Lab
Roc
Bec
Res
and
wer
lab

wit
des
was
of

usi
sta
(P

ß-N
pur
ß-N
cor
pur
int
ins

Boehringer Mannheim Biochemicals, Indianapolis, IN. Affigel HZ beads and all electrophoresis reagents were from BioRad Laboratories, Richmond, CA. ORIGM was from IGEM Inc., Rockville, MD. Immobilon was from Millipore Corporation, Bedford, MA. RIBI adjuvant was from RIBI Immunochemical Research Inc., Hamilton, MO. Sodium hypoxanthine, aminopterin, and thymidine (HAT) and Dulbecco's Modified Eagle Media (DME) were from Sigma. Bovine fetal calf serum was from HyClone laboratories, Logan, UT.

Enzyme Assay-ß-mannosidase activity was assayed at pH 5.0 with 4-methylumbelliferyl ß-D-mannopyranoside as previously described (Jones et al., 1984). One unit of enzyme activity was defined as that amount of enzyme which hydrolysed one umol of substrate/hr at 37 °C.

Protein Determinations-Protein concentrations were measured using bicinchoninic acid (BCA) and bovine serum albumin as a standard (Smith et al., 1985) with a BCA protein assay kit (Pierce, Rockford, IL).

Production of Monoclonal Antibodies Against Caprine ß-Mannosidase-Outbred mice were immunized with a partially purified preparation of caprine ß-mannosidase. Caprine ß-mannosidase was purified 5,000 fold from goat kidney using conventional chromatography (Frei et al., 1988). The partially purified enzyme was emulsified in RIBI adjuvant and injected into outbred mice according to the manufacturer's instructions. The spleens from mice with serum titers capable

of precipitating one unit of caprine ß-mannosidase activity per microliter of serum were removed and the splenocytes were fused with CRL 1580 cells (Kennett, 1980). The fused cells were resuspended in HAT-DME medium supplemented with 20% bovine fetal calf serum and 5% ORIGM hybridoma cloning factor. The fused cells were cloned by limiting dilution. Approximately two weeks following the fusion, the supernatants were screened in a dot immunobinding assay (Campbell, 1984) using a partially purified (2,000 fold) ß-mannosidase enzyme preparation as antigen. The positive supernatants were screened for the ability to precipitate ß-mannosidase activity. Positive clones were recloned by limiting dilution.

Extraction and Con A-Sepharose Chromatography-Goat kidney (3 kg) was minced and homogenized in 600 g batches with a Waring blender (3x30 s) in one volume (600 ml) of extraction buffer (50 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 10% (v/v) glycerol, 10 mM citrate (pH 5.5), 0.5 mg/L leupeptin, 0.7 mg/L pepstatin and 0.02% $NaN_3$) at 4 °C. The crude homogenate was centrifuged (16,000 g for 30 min) at 4 °C and the supernatant saved. The resuspended pellets were subjected to a second cycle of homogenization/centrifugation and the supernatant was pooled with that of the first cycle. The pool of high speed supernatant was mixed with 500 ml of Con A-Sepharose for two hours at room temperature with gentle agitation. The Con A-Sepharose/crude extract slurry was filtered and the Con A-Sepharose was washed with 12 liters of extraction buffer

over a period of 10-20 minutes. The washed resin was then transferred to a 5x45 cm column. The glycoproteins were eluted from the resin with 1.2-1.4 L of elution buffer (0.7 M methyl-α-glucoside, 0.55 M NaCl, 0.01 M citrate (pH 5.5), 0.02% $NaN_3$, 10% glycerol, 0.5 mg/L leupeptin and 0.7 mg/L pepstatin) at a flow rate of 100 ml/hr. The pool of eluted glycoproteins was diluted with one volume of saturated ammonium sulfate (25 °C) and immediately centrifuged at 16,000 g for 30 minutes at 4 °C. The ammonium sulfate pellets were stored at -80 °C prior to immunoaffinity chromatography.

Immunoaffinity Purification-Monoclonal antibody (MAb) produced by the hybridoma cell line 43F10S was purified on a Protein A-agarose column (Harlow and Lane, 1988). The purified antibody (15 mg) was periodate-activated and reacted with the amine groups of 7 mL of Affi-gel HZ beads (Harlow and Lane, 1988). Ammonium sulfate pellets were resuspended in supplemented Tris-buffered saline (TBS) (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mg/L leupeptin, 0.7 mg/L pepstatin and 0.02% $NaN_3$) to a final protein concentration of 20 mg/ml. The resuspended pool was delipidated with a 1:3 ratio of carbon tetrachloride to sample. A preclearing column containing 10 ml of agarose beads was placed in line prior to the affinity column and delipidated sample was loaded at a flow rate of 20 ml/h at 4 °C. After loading, the affinity column was washed with 20 column volumes of TBS containing 1% (v/v) Triton X-100

followed by 20 column volumes of TBS alone. The enzyme was eluted with a total volume of 40 ml McIlvaine's (Sober, 1968) citric acid-phosphate buffer (pH 6.25) that contained stepwise (10 ml/step) increases in deionized urea (5,6,7 and 8 M). Eluate was collected in dialysis tubing that was suspended in a beaker of TBS. Dialysis was continued for 24 hrs at 4 °C with several changes of buffer.

Analytical Scale Immunoaffinity Purification of ß-Mannosidase from Normal and Affected Goat Kidney Tissue-Equivalent amounts of age-matched normal (55 g) and affected (52 g) tissue were homogenized in 200 ml of extraction buffer as described above. The high speed supernatants (normal and affected) were passed through Con A-Sepharose columns (20 mL) until the ß-mannosidase activity in the normal pool (>95%) had been absorbed to the resin. The columns were washed with ten column volumes of extraction buffer and eluted with 125 ml of elution buffer at a flow rate of 25 ml/h. The pool of eluted glycoproteins was precipitated with ammonium sulfate as described for the extraction procedure and the pellets were dissolved in 10 ml of supplemented TBS. These pools of dissolved protein (normal and affected) were repeatedly passed through 43F10S immunoaffinity columns (0.5 ml) until all the ß-mannosidase activity in the normal pool had been adsorbed to the resin. The columns were washed as described for the affinity purification procedure. The enzyme was eluted with 4 ml of McIlvaine's (Sober, 1968)

citric acid-phosphate buffer (pH 3.0) containing stepwise (1 ml/step) increases in deionized urea (1, 1.5, 2 and 4 molar). One microliter of each normal fraction was diluted 100 fold into McIlvaine's citric acid-phosphate buffer (pH 5.0) immediately following elution from the column and assayed for enzyme activity. Using this elution procedure 23% of the ß-mannosidase activity in the Con A pool (from normal tissues) was recovered in the immunoaffinity pool. The eluted material was stored at -80 °C prior to SDS-PAGE analysis.

High Performance Liquid Chromatography-A Waters 625 LC system equipped with a Lambda-Max 481 spectrophotometer was used with the following columns: a Pharmacia Mono S HR 5/5 (cation-exchange) column and a Pharmacia Superose 12 HR 10/30 (gel filtration) column. To concentrate the protein prior to Mono S chromatography the immunoaffinity pool (45 ml) was dialyzed into 1.25 M ammonium sulfate and bound to a 1.0 ml low pressure TSK butyl column. The bound protein was eluted with a 10 mM Tris-Cl buffer (pH 6.8) in a 4 ml volume with essentially complete recovery of protein and activity. This pool was dialyzed into a citrate buffer (10 mM citrate, pH 5.5, 50 mM NaCl and 10% glycerol) and loaded onto the Mono S column. The enzyme was eluted with a NaCl gradient as described previously (Frei et al., 1988). For gel filtration the Superose 12 column was equilibrated with 150 mM-ammonium carbonate buffer (pH 8.0) and 200 ul of the immunoaffinity pool was injected. The fractions (0.5 ml) were lyophilized and

resuspended in 50 ul of SDS-PAGE sample buffer.

SDS-PAGE Analysis-Gel electrophoresis, gel staining, transfers and Western analysis were done essentially as described in Current Protocols in Molecular Biology (Ausubel et al., 1987). The SDS-PAGE sample buffer contained 2.5 % (v/v) 2-mercaptoethanol. The second antibodies used in the Western analysis were alkaline phosphatase conjugated goat anti-mouse IgG and goat anti-rabbit IgG.

Peptide Mapping by Limited Proteolysis in SDS-PAGE-Peptide mapping was carried out essentially as described by Cleveland and colleagues (Cleveland et al., 1977), as adapted by Polakis and Wilson (Polakis and Wilson, 1984). Modifications of the procedure of Polakis and Wilson are described in the legend of Fig. 2.

Glycosylation Studies-Immunoaffinity purified ß-mannosidase (20 uL, 0.1 ug/ul) was heated (65 °C, 15 min) in the presence of 0.15% SDS and 0.3 M ß-mercaptoethanol. Following denaturation the sample was diluted by the addition of 15 ul of 0.077 M potassium phosphate (pH 7.0), 1.5% N-octylglucoside, 0.065 M EDTA. N-glycosidase F (0.3 units) or Endoglycosidase H (0.004 units) was added and the sample was incubated at 37 °C for 12 hrs.

Production of Polyclonal Antiserum-Immunoaffinity purified caprine ß-mannosidase (300 ug) was subjected to preparative SDS-PAGE. Following electrophoresis the gel was stained with an aqueous solution of Coomassie blue (0.5 g/L) and destained

in water. Gel slices containing the 80 or the 90 kDa peptide were processed by repeatedly working the acrylamide through a syringe until it could be easily passed through a 21-gauge needle. The processed acrylamide was emulsified in incomplete Freund's adjuvant prior to injection (Harlow and Lane, 1988). The gel slice containing the 90 kDa peptide was used to immunize and boost two New Zealand White rabbits. One of the rabbits produced antiserum that reacts with the 90 and 100 kDa peptides plus the 80 kDa peptide which was very likely a minor contaminant in the gel slice. This antiserum is referred to throughout the text as anti-80/90/100. The gel slice containing the 80 kDa peptide was used to immunize and boost one rabbit and produced antiserum that reacts with only the 80 kDa peptide. This antiserum is referred to throughout the text as anti-80.

## RESULTS

Production and Characterization of MAbs Against Caprine ß-Mannosidase-Screening of the hybridomas, generated from one mouse spleen, identified eight hybridoma clones that produced antibodies capable of precipitating ß-mannosidase activity. When used to probe a Western blot of partially purified ß-mannosidase (2,000 fold) three MAbs failed to produce detectable signals with the denatured protein but three others

identified a 90 and a 100 kDa peptide. MAb 44D9 gave the strongest signal in this analysis (data not shown). Evaluation of the specificity of MAb 43F10S towards a panel of lysosomal hydrolases revealed that the antibody could precipitate greater than 95% of the ß-mannosidase activity in goat plasma and crude kidney extracts without precipitating detectable levels of α-mannosidase, α-glucosidase, α-fucosidase or ß-hexosaminidase.

Immunoaffinity Purification of Lysosomal ß-Mannosidase-Following Con A-Sepharose chromatography (Frei et al., 1988), ß-mannosidase was purified by immunoaffinity chromatography using the MAb 43F10S. The immunoaffinity pool contains primarily three peptides (Fig. 1, lane A) as assessed by Coomassie blue stained SDS-PAGE. Two of these peptides (100 and 90 kDa) react with the MAb 44D9 and the third peptide (a broad 80 kDa band) does not (Fig. 1 lane B). A small amount of mouse IgG eluted from the immunoaffinity column is detected by the goat anti-mouse IgG serum (Fig. 1 lane C). To further address the degree of relatedness between the 100, 90 and 80 kDa peptides they were subjected to peptide mapping by limited proteolysis in SDS-PAGE (Fig. 2). Limited proteolysis of the 100 and 90 kDa peptides produced very similar peptide patterns. The 80 kDa peptide pattern does not appear to be related to that of either the 100 or 90 kDa peptides.

HPLC Purification and Characterization-For additional purification of the enzyme and to determine whether the 80 kDa

peptide is associated with, or required for, ß-mannosidase activity, the immunoaffinity pool was subjected to HPLC chromatography.

Gel filtration of immunoaffinity purified ß-mannosidase produced two protein peaks (Data not shown). The major peak contained approximately 90% of the protein and all of the ß-mannosidase activity. SDS-PAGE analysis of selected fractions revealed that the predominant peptides in the fractions corresponding to enzymatic activity were the 90 and 100 kDa peptides. A major portion of 80 kDa peptide eluted in a broad peak, prior to the enzyme activity, apparently as an oligomer or aggregate.

The elution position of the 100 and 90 kDa peptides from the cation exchange column (Fig. 3B) also corresponds to the activity profile (Fig. 3A) while the 80 kDa peptide eluted earlier. ß-Mannosidase activity is apparently associated with both the 90 and 100 kDa peptides which are partially resolved by Mono S chromatography. Western blot analysis of fraction 18 was performed to identify the smaller peptides which were present in this preparation (data not shown). The 35 kDa peptide whose elution position also corresponds with enzyme activity reacts with anti-80/90/100 polyclonal serum. There is no reactivity of any of the peptides in fraction 18 with anti-80 polyclonal serum while anti-mouse IgG reacted with the 58 and 28 kDa peptides. Therefore, the 58 and 28 kDa peptides are mouse IgG which co-elute with the 90 kDa peptide from Mono

S chromatography. The 35 kDa peptide is immunologically related to the 90 and 100 kDa peptides and may be the result of a small amount of protein degradation.

Carbohydrate Composition of ß-Mannosidase-The level and type of glycosylation of the 100 and 90 kDa peptides was examined using Endoglycosidase H and N-glycosidase F (Fig. 4). N-Glycosidase F which removes all N-linked oligosaccharides from glycoproteins, reduced the molecular size of the 100 and 90 kDa peptides to 91 and 86 kDa respectively (Fig. 4 lane 3). Glycosylation of the 90 kDa peptide is mainly high mannose since Endoglycosidase H digestion yielded an approximately 86 kDa peptide (Fig 4, lane 1). The 100 kDa peptide is more heavily glycosylated and contains complex-type oligosaccharides since Endoglycosidase H digestion reduces the size of the 100 kDa peptide to approximately 96 kDa.

Comparative Analysis of Affinity Purified ß-Mannosidase from Normal and Affected Tissues-To characterize the defect in goats affected with ß-mannosidosis, kidneys from affected and control animals were subjected to the first two steps of the purification procedure. Equivalent amounts of age-matched normal (55 g) and affected (52 g) kidney tissue were homogenized, bound and eluted from Con A-Sepharose. The pools of glycoproteins recovered from this step (normal and affected) contained almost identical levels of protein and α-mannosidase activity. Nearly equivalent amounts of the 80 kDa peptides are present in the Con A-Sepharose pool from affected

and normal kidney tissue prior to and following immunoaffinity-depletion (Fig. 5A). The pools of glycoproteins (normal and affected) from the Con A-Sepharose step were subjected to immunoaffinity purification using two identical analytical sized columns (0.5 ml of resin). Following elution, equivalent volumes of the normal and affected eluate were analyzed by SDS-PAGE. Both silver staining and Western analyses of the immunoaffinity pool from affected tissues indicate that the 90 and 100 kDa peptides are absent (Fig. 5B and 5C).

## DISCUSSION

An immunoaffinity purification procedure has been developed to prepare purified goat kidney lysosomal ß-mannosidase in high yield. This protocol, summarized in Table 1, produces an enzyme preparation with a specific activity of 1,190 units/mg of protein with a 26% yield and a 120,000-fold purification. When assessed by silver stained SDS-PAGE or by Western analysis, two peptides (100 and 90 kDa) predominate and these peptides are associated with ß-mannosidase activity in subsequent chromatography. Activity is associated with both the 90 and 100 kDa peptides which were partially resolved by Mono S chromatography. The 90 and 100 kDa peptides both react with anti-ß-mannosidase monoclonal antibodies and produce very

similar peptide patterns when subjected to limited proteolysis. Deglycosylation of the peptides reveals that they differ in size by approximately 5 kDa. The nature of the relationship between these two peptides is currently undetermined. A precursor/product or alternative processing relationship seems plausible and would be consistent with our findings.

When extracts of kidney tissue from goats with ß-mannosidase deficiency are subjected to Con A-Sepharose and immunoaffinity chromatography, the 90 and 100 kDa peptides are not detected in the immunoaffinity eluate, as judged by silver staining and Western analysis. This implies that these peptides are either absent in affected tissue or they are structurally altered in such a way that either the Concanavalin A or the MAb 43F10S does not bind to them. The absence of both of these peptides in animals affected by this autosomal recessive disease further substantiates the hypothesis that these peptides have a precursor/product or alternative processing relationship. An alternative hypothesis, that these peptides represent two nonallelic isozymes, is unlikely since caprine ß-mannosidosis is inherited in a simple autosomal recessive manner.

The major contaminants following immunoaffinity chromatography are an 80 kDa peptide and the light and heavy chains of mouse IgG. The 80 kDa peptide is almost completely resolved from the activity profile by the process of gel

filt
abun
perc
colu
in a
A-Se
vari
vivo
sinc
G
puri
kidn
abse
anim
thei

A
Depa
hybr
woul
work
work

filtration or Mono S chromatography. The 80 kDa peptide is abundant in the Con A-Sepharose pool and only a very small percent of the peptide is retained by the immunoaffinity column. The amount and size of the 80 kDa peptide is unchanged in affected tissues as detected by Western analysis of the Con A-Sepharose pool. Another peptide of 35 kDa is present in variable amounts and probably represents an in vitro or in vivo proteolytic fragment of the larger 90 or 100 kDa peptide since it is immunologically related to them.

Goat lysosomal ß-mannosidase can now be reproducibly purified to near homogeneity. The enzyme preparation from kidney consists of two related peptides of 100 and 90 kDa. The absence or modification of these peptides in tissue from animals with ß-mannosidase deficiency further substantiates their identity.

Acknowledgments-I would like to thank Peggy Bull of the Department of Pathology at Michigan State University for the hybridoma production and her assistance with cell culture. I would also like to thank Christine Traviss, my part-time co-worker, for her assistance throughout the duration of this work.

## REFERENCES

Abbitt, B., Jones, M.Z., Kasari, T.R., Storts, R.W., Templeton, J.W., Holland, P.S. and Castenson, P.E. ß-Mannosidosis in twelve Salers calves. J.Am.Vet.Med.Assoc. 198:109-113, 1991.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. Current protocols in molecular biology, New York: Greene Publishing Associates and Wiley-Interscience (John Wiley & Sons), 1987.

Bernard, M., Sioud, M., Percheron, F. and Foglietti, M.J. ß-Mannosidase in human serum and urine. A comparative study. Internat.J.Biochem. 18:1065-1068, 1986.

Boyer, P.J., Jones, M.Z., Nachreiner, R.F., Refsal, K.R., Common, R.S., Kelley, J. and Lovell, K.L. Caprine ß-mannosidosis: Abnormal thyroid structure and function in a lysosomal storage disease. Lab.Invest. 63:100-106, 1990.

Bryan, L., Schmutz, S., Hodges, S.D. and Snyder, F.F. Bovine ß-mannosidase deficiency. Biochem.Biophys.Res.Comm. 173:491-495, 1990.

Campbell, A.M. Monoclonal antibody technology, Amsterdam: Elsevier, 1984, pp. 58-59.

Cava
M.Z.
Will
prop
nonl

Clev
U.K.
sulf
252:

Coop
Norc
epil
1991

Daws
manr

Dorl
de J
van
loss

Cavanagh, K., Fisher, R.A., Legler, G., Herrchen, M., Jones, M.Z., Julich, E., Sewell-Alger, R.P., Sinnott, M.L. and Wilkinson, F.E. Goat Liver ß-mannosidases: Molecular properties, inhibition and inactivation of the lysosomal and nonlysosomal forms. Enzyme 34:75-82, 1985.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J.Biol.Chem. 252:1102-1106, 1977.

Cooper, A., Wraith, J.E., Savage, W.J., Thornley, M. and Noronha, M.J. ß-Mannosidase deficiency in a female infant with epileptic encephalopathy. J.Inherited Metab.Dis. 14:18-22, 1991.

Dawson, G. Evidence for two distinct forms of mammaliam ß-mannosidase. J.Biol.Chem. 257:3369-3371, 1982.

Dorland, L., Duran, M., Hoefnagels, F.E.T., Breg, J.N., Fabery de Jonge, H., van Eghen-Cransberg, K., van Sprang, F.J. and van Diggelen, O.P. ß-Mannosidosis in two brothers with hearing loss. J.Inherited Metab.Dis. 11:255-258, 1988.

Frei, J.I., Cavanagh, K., Fisher, R.A., Hausinger, R.P., Dupis, M., Rathke, E.J.S. and Jones, M.Z. Partial purification of goat kidney ß-mannosidase. Biochem.J. 249:871-875, 1988.

Harlow, E. and Lane, D. Antibodies: A laboratory manual, New York: Cold Spring Harbor Laboratory, 1988.

Healy, P.J., Seaman, J.T., Gardner, I.A. and Sewell, C.A. ß-Mannosidase deficiency in anglo nubian goats. Aust.Vet.J. 57:504-507, 1981.

Iwasaki, Y., Tsuji, A., Omura, K. and Suzuki, Y. Purification and characterization of ß-mannosidase from human placenta. J.Biochem. 106:331-335, 1989.

Jolly, R.D., Thompson, K.G., Bayliss, S.L., Vidler, B.M., Orr, M.B. and Healy, P.J. ß-Mannosidosis in a Salers calf: A new storage disease of cattle. J.New Zealand Vet.Assn. (in press), 1991.

Jones, M.Z. and Dawson, G. Caprine ß-mannosidosis: Inherited deficiency of ß-mannosidase. J.Biol.Chem. 256:5185-5188, 1981a.

Jones, M.Z. and Laine, R.A. Caprine oligosaccharide storage disease: Accumulation of ß-mannosyl (1-4) ß-N-

acetylglucosaminyl (1-4) ß-N-acetylglucosamine in brain. J.Biol.Chem. 256:5181-5184, 1981b.

Jones, M.Z., Cunningham, J.G., Dade, A.W., Alessi, D.W., Mostosky, U.V., Vorro, J.R., Benitez, J.T. and Lovell, K.L. Caprine ß-mannosidosis: Clinical and pathological features. J.Neuropath.Exp.Neurol. 42:268-285, 1983.

Jones, M.Z., Rathke, E.J.S., Cavanagh, K. and Hancock, L.W. ß-Mannosidosis: Prenatal biochemical and morphological characteristics. J.Inherited Metab.Dis. 7:80-85, 1984.

Jones, M.Z., Rathke, E.J.S., Gage, D.A., Costello, C.E., Murakami, K., Ohta, M. and Matsura, F. Oligosaccharides accumulated in the bovine ß-mannosidosis kidney. J.Inherited Metab.Dis. (in press), 1991.

Kennett, R.H. Fusion by centrifugation of cells suspended in polyethylene glycol. In: Monoclonal Antibodies. Hybridomas: A new dimension in biological analyses, edited by Roger H. Kennett, Thomas J. McKearn and Kathleen B. Bechtol, New York: Plenum Press, 1980, pp. 365-367.

Kleijer, W.J., Hu, P., Thoomes, R., Boer, M., Huijmans, J.G.M., Blom, W., van Diggelen, O.P., Seemanova, E. and Macek, M. ß-Mannosidase deficiency: Heterogeneous manifestations in

the first female patient and her brother. J.Inherited Metab.Dis. 13:867-872, 1990.

Kyosaka, S., Murata, S., Nakamura, F. and Tanaka, M. Purification and kinetic properties of guinea pig liver ß-mannosidase. Chem.Pharm.Bull. 33:256-263, 1985.

Lovell, K.L. and Boyer, P.J. Dysmyelinogenesis in caprine ß-mannosidosis: Ultrastructural and morphometric studies in fetal optic nerve. Int.J.Devl.Neuroscience 5:243-253, 1987.

Matsuura, F., Laine, R.A. and Jones, M.Z. Oligosaccharides accumulated in the kidney of a goat with ß-mannosidosis: Mass spectrometry of intact permethylated derivatives. Arch.Biochem.Biophys. 211:485-493, 1981.

Matsuura, F., Jones, M.Z. and Frazier, S.E. Structural analysis of the major caprine ß-mannosidosis urinary oligosaccharides. Biochim.Biophys.Acta 759:67-73, 1983.

McCabe, N.R., Biliter, W. and Dawson, G. Preferential inhibition of lysosomal ß-mannosidase by sucrose. Enzyme 43:137-145, 1990.

McCabe, N.R. and Dawson, G. A study of the heterogenous structure of guinea pig lysosomal ß-mannosidase using a

polyclonal antibody. Biochim.Biophys.Acta 1077:133-140, 1991.

Noeske, C. and Mersmann, G. ß-D-Mannosidase from human placenta: Properties and partial purification. Hoppe-Seyler'sZ.Physiol.Chem. 364:1645-1651, 1983.

Pearce, R.D., Callahan, J.W., Little, P.B., Armstrong, D.T., Kiehm, D. and Clarke, J.T.R. Properties and prenatal ontogeny of ß-mannosidase in selected goat tissues. Biochem.J. 243:603-609, 1987.

Polakis, P.G. and Wilson, J.E. Proteolytic dissection of rat brain hexokinase: Determination of the cleavage pattern during limited digestion with trypsin. Arch.Biochem.Biophys. 234:341-352, 1984.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K. Goeke, N.M., Olson, B.J. and Klenk, D.C. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85, 1985.

Sober, H.A. Buffer solutions. In: CRC handbook of biochemistry: Selected data for molecular biology, Cleveland: The Chemical Rubber Company, 1968, pp. J-195-J-198.

Tjoa, S., Wenger, D.A. and Fennessey, P.V. Quantitative analysis of disaccharides in the urine of ß-mannosidosis patients. J.Inherited Metab.Dis. 13:187-194, 1990.

van Pelt, J., Hokke, C.H., Dorland, L., Duran, M., Kamerling, J.P. and Vliegenthart, J.F.G. Accumulation of mannosyl-ß(1-4)-N-acetylglucosamine in fibroblasts and leukocytes of patients with a deficiency of ß-mannosidase. Clin.Chim.Acta 187:55-60, 1990.

Wenger, D.A., Sujansky, E., Fennessey, P.V. and Thompson, J.N. Human ß-mannosidase deficiency. N.Engl.J.Med. 315:1201-1205, 1986.

TABLE I

Summary of the purification of ß-mannosidase from normal goat kidney

| Purification step | Total activity | Total protein | Specific activity | Yield | Purification |
|---|---|---|---|---|---|
| | units | mg | units/ mg protein | % | -fold |
| High speed supernatant | 2,430 | 243,000 | 0.010 | 100 | 1 |
| Con A-Sepharose chromatography | 1,700 | 4,080 | 0.417 | 70 | 42 |
| Immunoaffinity chromatography | 825 | 1.3 | 635 | 34 | 63,000 |
| HPLC: Mono S chromatography | 643 | 0.54 | 1,190 | 26 | 120,000 |

Fig. 1. **Immunoaffinity purified ß-mannosidase.** Immunoaffinity purified ß-mannosidase (20 ug/lane) was subjected to SDS-PAGE (7.5% acrylamide) and protein was visualized with Coomassie blue (lane a). Immunoaffinity purified ß-mannosidase (20 ug/lane) was subjected to SDS-PAGE (10% acrylamide) and transferred to Immobilon-P (lanes b and c) and visualized with the MAb 44D9 (lane b) or goat anti-mouse IgG antiserum (lane c). Sizes indicated are in kDa.

Fig. 1

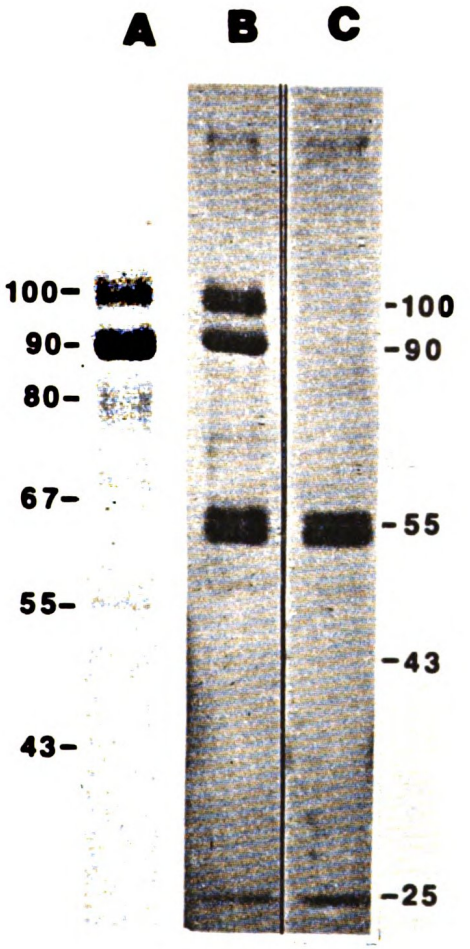


Fig. 2. **Peptide mapping by limited proteolysis in SDS-PAGE.** Affinity purified ß-mannosidase (30 ug) was subjected to SDS-PAGE (10% acrylamide). Following electrophoresis, the gel was stained with Coomassie blue (0.5 g/L $H_20$) and destained in water. Gel slices containing the 80, 90 and 100 kDa peptides were excised from the gel and placed in separate wells of a second SDS-PAGE (15% acrylamide) gel. Staphylococcus aureus V8 protease (20 ng/lane) suspended in sample buffer was added to each well. Electrophoresis was carried out until the tracking dye reached the interface between the stacking and separating gels. The power was turned off for 30 min, allowing proteolysis to occur within the gel, after which the electrophoresis was continued. The protein bands were visualized by Coomassie blue/silver staining. Sizes indicated are in kDa.

Fig. 2

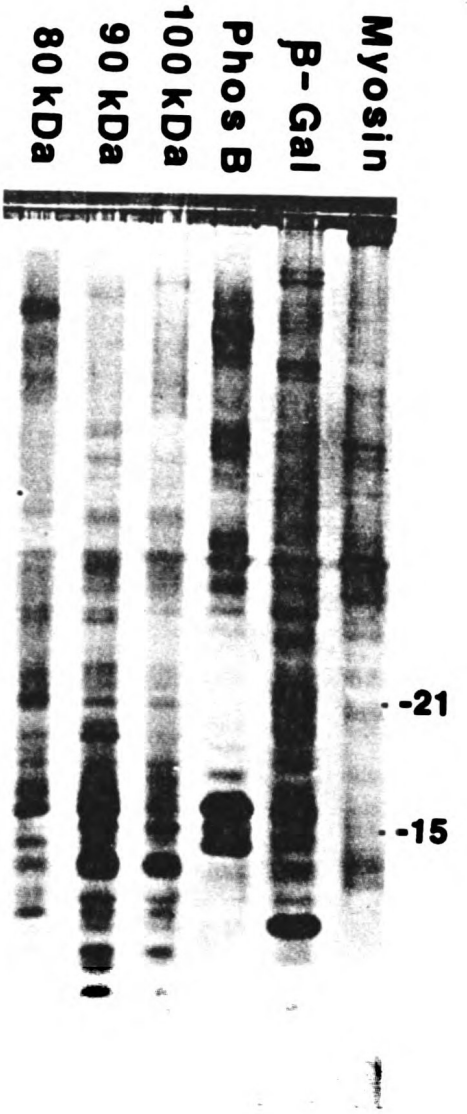
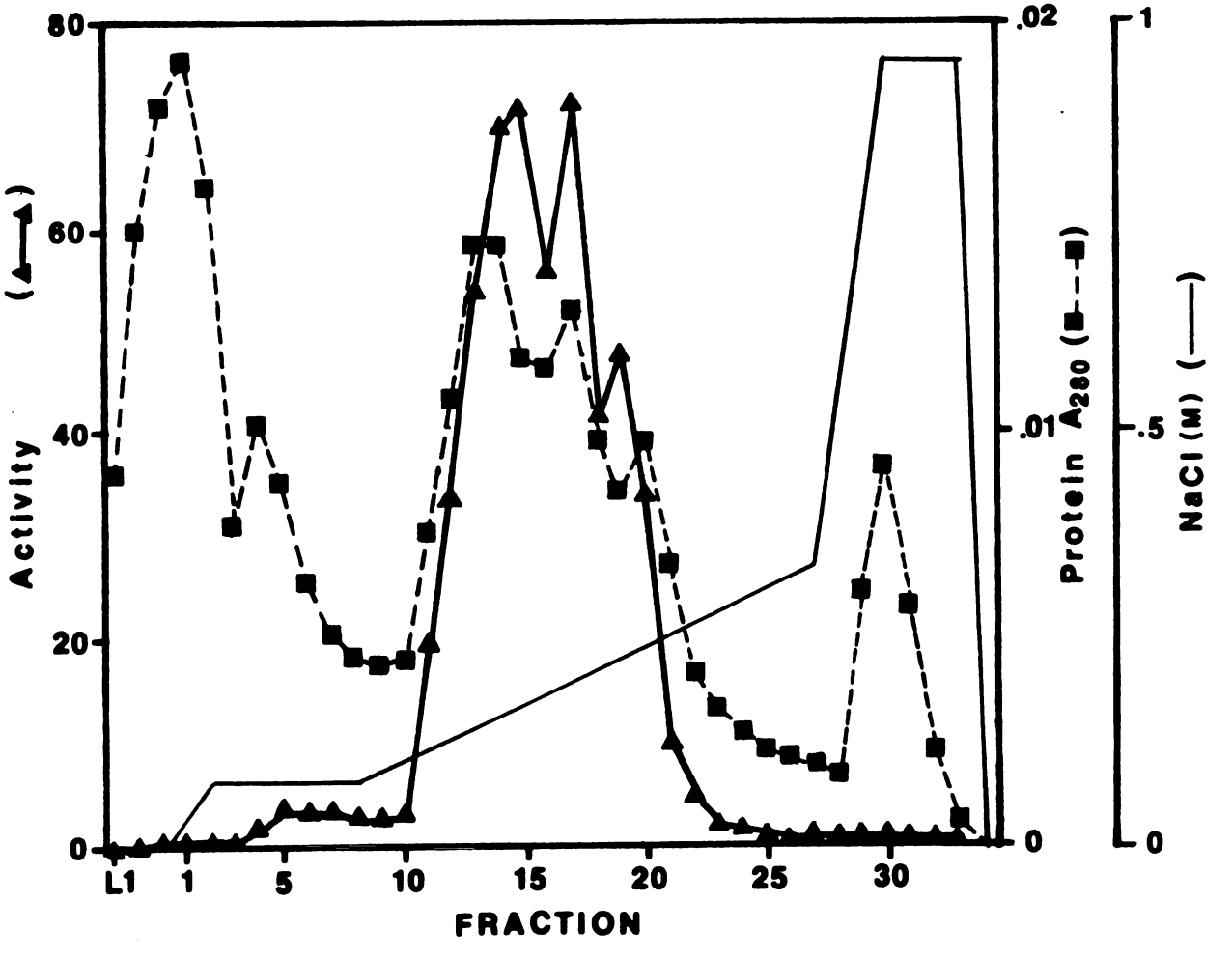


Fig. 3. **Cation-exchange chromatography of ß-mannosidase.** Panel A: The concentrated and dialyzed immunoaffinity pool (6 mL/825 units/1.3 mg) was applied to a Mono S HR 5/5 column and eluted with a multi-step NaCl gradient. L1-L3 are 2 mL fractions collected during column loading, 1 mL fractions were collected during elution. Enzyme activity is total units/fraction and protein was monitored at $A_{280}$. Panel B: Aliquots (20 uL) from selected fractions (indicated above the gel) were subjected to SDS-PAGE (10% acrylamide) analysis. Protein bands were visualized by silver staining. Molecular mass standards (in kDa) are indicated on the left of the figure.

Fig. 3A


80
60
40
20
0
Activity
.02
.01
0
Protein $A_{280}$
1
.5
0
NaCl (M)
L1
1
5
10
15
20
25
30
FRACTION

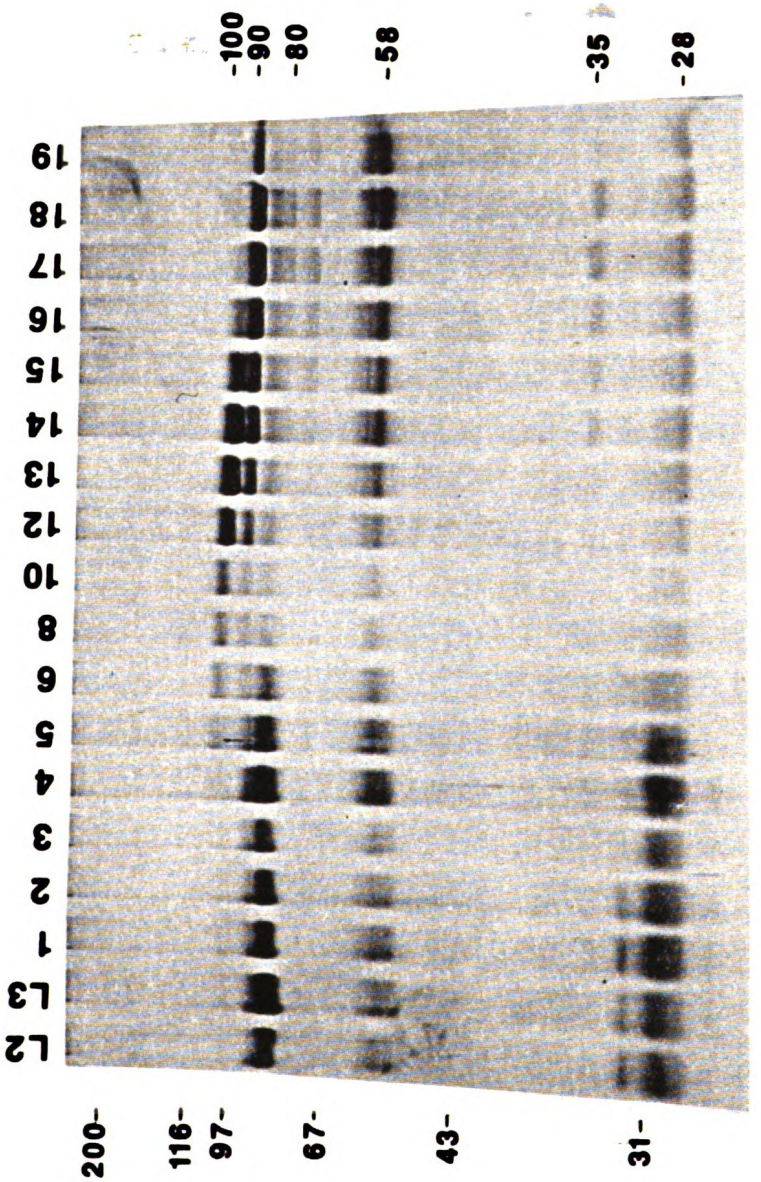

-100
-90
-80
-58
-35
-28
19
18
17
16
15
14
13
12
10
8
6
5
4
3
2
1
L3
L2
200-
116-
97-
67-
43-
31-

Fig. 3B

Fig. 4 **Glycosylation of affinity purified ß-mannosidase.** Immunoaffinity purified ß-mannosidase (2 ug) was deglycosylated with Endoglycosidase H (lane 1), no glycosidase (lane 2) or N-glycosidase F (lane 3). The digestion mixtures were resolved on SDS-PAGE (10% acrylamide) and protein bands were visualized by silver staining. Molecular weight standards (in kDa) are indicated on the left of the figure.

Fig. 4

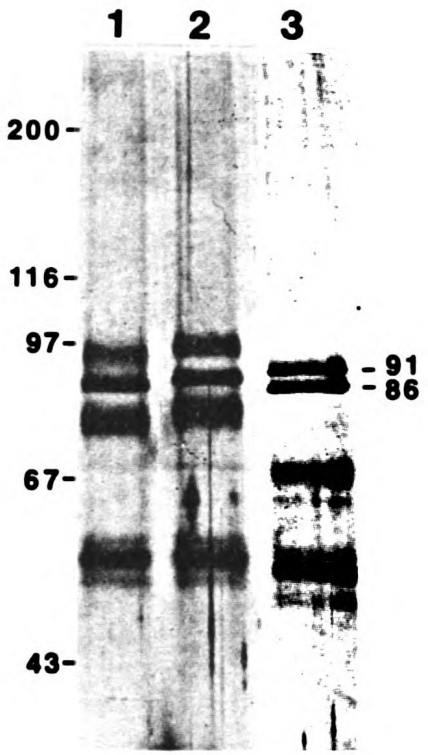


Fig. 5. **Comparative analysis of affinity purified ß-mannosidase from normal and affected goat tissues.** Kidney tissue from normal (N) and ß-mannosidase deficient (D) goats was subjected to Con A-Sepharose and immunoaffinity chromatography. Equal volumes of the normal and affected Con A-Sepharose eluate prior to (panel A1) and following immunoaffinity depletion (panel A2) were resolved on SDS-PAGE (5-15% acrylamide), transferred to Immobilon-P and probed with anti-80 polyclonal serum. Equal volumes of the normal and affected immunoaffinity pools were subjected to SDS-PAGE (5-15% acrylamide) analysis. Protein was detected by silver staining (panel B) or transferred to Immobilon-P and detected with anti-80/90/100 polyclonal serum (panel C). Molecular weight standards (in kDa) are indicated on the right of the figures.

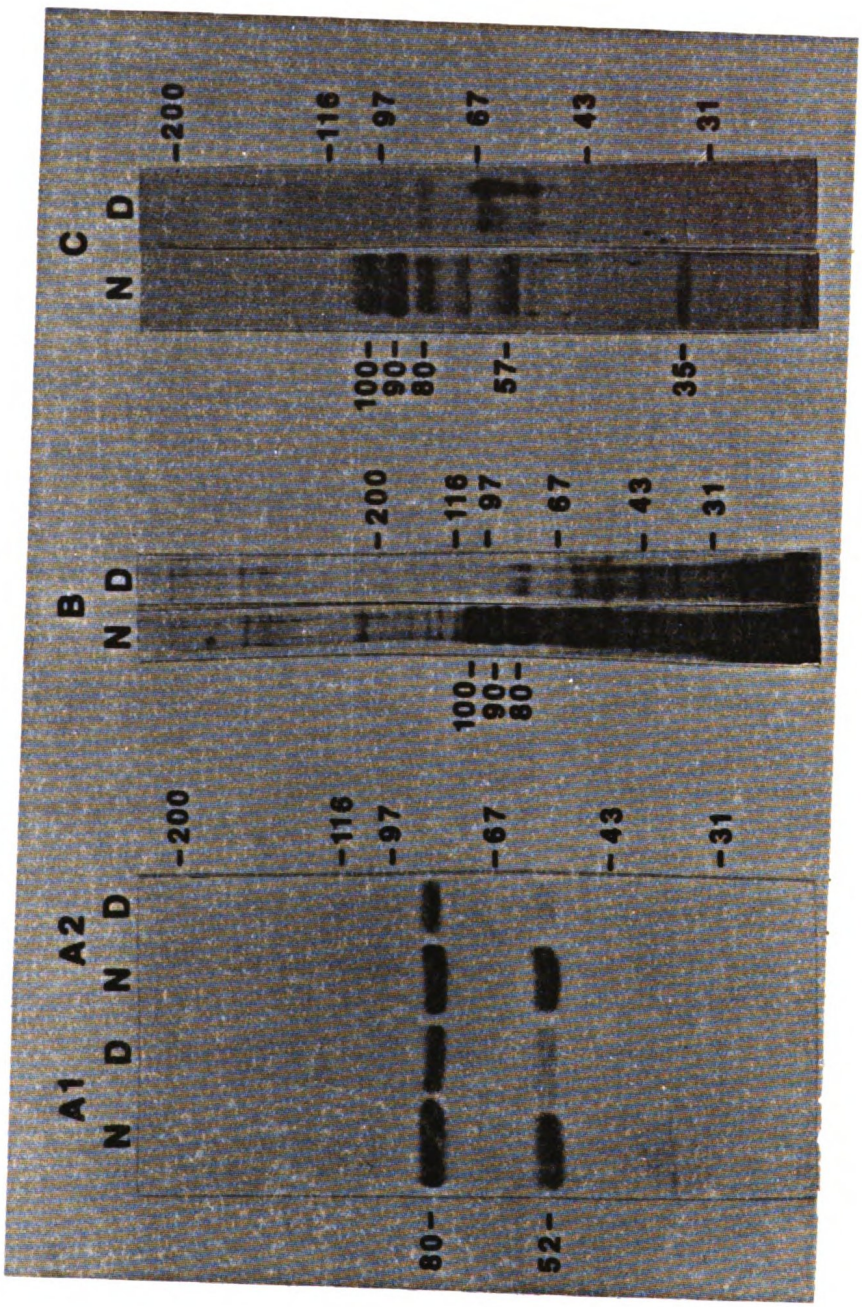



Fig. 5

# Bovine ß-Mannosidase: Purification, Characterization and Peptide Sequence Analysis

## ABSTRACT

Lysosomal ß-mannosidase was purified 160,000 fold in 24% yield from bovine kidney using a four step purification procedure which included Concanavalin A-Sepharose, immunoaffinity, TSK-butyl and cation-exchange chromatography. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining, the purified enzyme preparation consists of three peptides (80, 100 and 110 kDa). The 110 and 100 kDa peptides are consistently associated with ß-mannosidase activity in chromatography subsequent to immunoaffinity purification. Removal of N-linked carbohydrate from the 110 and 100 kDa peptides reduces their molecular weight to 91 and 86 kDa, respectively. Kidney tissue from calves with ß-mannosidase deficiency (ß-mannosidosis) does not contain detectable quantities of the 80, 100 or 110 kDa peptides as judged by monoclonal and polyclonal antibody reactivity.

Sequence analysis of three cyanogen bromide fragments of the 100 kDa bovine peptide yielded some sequence information. Peptides in two chromatography fractions were sequenced. One fraction yielded six residues of unambiguous sequence and the other fraction yielded nine residues of sequence from two different N-termini.

# INTRODUCTION

At least five different lysosomal glycosidases act in a progressive manner to degrade asparagine-linked oligosaccharides from their non-reducing end. The absence of any one of these glycosidases results in the storage and excretion of the undegraded substrate(s). Genetic disorders associated with ß-mannosidase deficiency (ß-mannosidosis) have been described in Nubian goats (Jones and Dawson, 1981a; Healy et al., 1981; Jones et al., 1984; Pearce et al., 1987), Salers cattle (Abbitt et al., 1991; Jolly et al., 1991; Bryan et al., 1990), and humans (Cooper et al., 1986; Wenger et al., 1986; Dorland et al., 1988; Cooper et al., 1991; Kleijer et al., 1990). The disaccharide Manß1-4GlcNAc, the trisaccharide Manß1-4GlcNAcß1-4GlcNAc and other more complex oligosaccharides accumulate in the tissues and urine of affected goats and calves (Jones et al., 1984; Jones and Laine, 1981b; Matsuura et al., 1981; Matsuura et al., 1983; Jones et al., 1991; Gage et al., 1992). In humans, the disaccharide Manß1-4GlcNAc is the major storage and excretion product (van Pelt et al., 1990; Tjoa et al., 1990).

The clinical features of ß-mannosidosis differ significantly between ruminants and humans. The clinical and pathological abnormalities of calves and goats with ß-mannosidosis are extremely similar (Hartley and Blakemore, 1973; Jones and Laine, 1981b; Jones et al., 1983; Jones and

Dawson, 1981a; Patterson et al., 1991; Abbitt et al., 1991; Jolly et al., 1991; Bryan et al., 1990; Jones et al., 1991; Lovell et al., 1991), with one noted exception. Calves, unlike goats, respond to loud noises and have intact auditory brainstem evoked potentials (Render et al., 1992). Goats and calves with ß-mannosidase deficiency are most notably characterized by severe neurological deficits associated with myelin abnormalities (Lovell and Jones, 1983; Jones, 1989; Boyer et al., 1990a; Boyer and Lovell, 1990b). Without immediate and intensive care following birth, affected calves and kids die within the first day or two of the neonatal period. Unlike goats and calves, clinical manifestations of ß-mannosidosis in humans can be mild and there is considerable clinical heterogeneity (Kleijer et al., 1990). In general, affected individuals are characterized by mental retardation, deafness and, in older patients, angiokeratoma.

ß-Mannosidase has been purified from guinea pig liver (Kyosaka, et al., 1985) and goat kidney (Sopher et al., 1992). The enzyme from guinea pig liver is reportedly a monomer of 110 kDa. The enzyme from goat kidney reportedly consists of two related peptides of 100 and 90 kDa.

To facilitate the isolation of quantities of purified ß-mannosidase suitable for polyclonal antibody production and peptide sequence analysis, I have undertaken the purification and characterization of this enzyme from bovine kidney. In this chapter I describe the purification and characterization

of bovine ß-mannosidase from normal and ß-mannosidase deficient tissues.

Peptide sequence analysis of this enzyme should facilitate the cloning of the ß-mannosidase gene. Cloning of this gene should further the characterization of the ß-mannosidase enzyme and the molecular characterization of the ß-mannosidosis disease mutations.

## EXPERIMENTAL PROCEDURES

Materials-Kidneys from mature cattle were obtained from Ada Beef in Ada, MI. Kidney tissue from calves with ß-mannosidosis was acquired from Bruce Abbitt at the Texas Veterinary Medical Diagnostic Laboratory, College Station, TX. All tissues were stored at -20 °C or -80 °C until needed. Concanavalin A-Sepharose 4B (Con A-Sepharose), 4-methylumbelliferyl ß-D-mannopyranoside, methyl-α-glucoside, cyanogen bromide and incomplete Freund's adjuvant were from Sigma, St. Louis, MO. Leupeptin, pepstatin, Endoglycosidase H and recombinant N-Glycosidase F were from Boehringer Mannheim Biochemicals, Indianapolis, IN. All electrophoresis reagents were from BioRad Laboratories, Richmond, CA. Immobilon-P was from Millipore Corporation, Bedford, MA. Extracti-gel was from Pierce, Rockford, IL.

Enzyme Assay-ß-Mannosidase activity was assayed at pH 5.0 with 4-methylumbelliferyl ß-D-mannopyranoside as previously described (Jones et al., 1984). One unit of enzyme activity was defined as that amount of enzyme which hydrolysed one umol of substrate/hr at 37 °C.

Protein Determinations-Protein concentrations were measured using bicinchoninic acid (BCA) and bovine serum albumin as a standard (Smith et al., 1985) with a BCA protein assay kit (Pierce, Rockford, IL).

Preparative Scale Purification of ß-Mannosidase-ß-Mannosidase was purified from bovine kidney as described for the goat enzyme (Sopher et al., 1992) with one modification. The immunoaffinity purified protein was eluted from the TSK-butyl resin using an ammonium sulfate gradient (1.25 - 0 molar) and the fractions with activity were pooled and then subjected to Mono-S chromatography as described for the goat enzyme (Frei et al., 1988).

Analytical Scale Purification of ß-Mannosidase-Bovine kidney tissues from normal and affected animals (60 g each) were subjected to a two step purification procedure (Con A-Sepharose followed by immunoaffinity purification) as described for the goat enzyme (Sopher et al., 1992).

SDS-PAGE Analysis-Gel electrophoresis, gel staining, transfers and Western analysis were performed essentially as described in Current Protocols in Molecular Biology (Ausubel et al., 1987). The SDS-PAGE sample buffer contained 2.5 %

(v/v) 2-mercaptoethanol. The second antibodies used in the Western analysis were alkaline phosphatase conjugated goat anti-rabbit IgG.

Production of Polyclonal Antiserum-Anti-bovine ß-mannosidase rabbit serum was produced as described for the goat enzyme (Sopher et al., 1992) with one modification. Mono-S pool (1 mg) was digested with recombinant N-Glycosidase F, as previously described for the goat enzyme (Sopher et al., 1992), prior to preparative SDS-PAGE. Removal of the carbohydrate enhanced the separation of the peptide of interest. One rabbit received three 50 ug injections of the SDS-PAGE purified and deglycosylated 86 kDa peptide (100 kDa prior to deglycosylation). The rabbit (Z59) produced antiserum specific for the 110 and 100 kDa peptides with no reactivity to the 80 kDa peptide. This antiserum is referred to throughout the text as anti-100/110.

Anti-80 rabbit serum, generated against the 80 kDa goat peptide (Sopher et al., 1992), was used in these studies to characterize the corresponding bovine peptide.

Anti-ß-hexosaminidase rabbit serum (alpha chain specific) was obtained from Richard Proia, National Institute of Health, Bethesda, MD.

Microsequencing-For the isolation of cyanogen bromide peptides, the concentrated Mono-S pool (2 mg) was deglycosylated as described (Sopher et al., 1992), cysteines were S-carboxamidomethylated (Fontana and Gross, 1986) and the

sample was subjected to preparative SDS-PAGE. Following electrophoresis the 86 kDa peptide (100 kDa peptide prior to deglycosylation) was electroeluted (Jacobs and Clad, 1986) and the extracted material was passed through Extracti-gel (Pierce) to remove the SDS. The extracted sample was digested with cyanogen bromide (Fontana and Gross, 1986) and the resulting peptides were separated by C8 reverse-phase HPLC (Tsai et al., 1988). The N-terminal amino acid sequences of the cyanogen bromide peptides were determined by automated gas-phase microsequencing and HPLC identification of phenylthiohydantoin amino acid derivatives (Hewick et al., 1981). The microsequencing was done at the Biomedical Research Core Facilities at the University of Michigan, Ann Arbor, MI.

## RESULTS

Purification of Bovine ß-Mannosidase-The immunoaffinity purification procedure, developed to purify goat kidney ß-mannosidase (Sopher et al., 1992), was used to prepare quantities of purified bovine ß-mannosidase suitable for polyclonal antibody production and peptide sequence analysis. The procedure, summarized in Table I, has produced an enzyme preparation with a specific activity of 2,000 units/mg of protein with a 24% yield and a 160,000 fold purification. When the profile of protein from the Mono-S column (Fig. 1A) is

assessed by Coomassie blue stained SDS-PAGE, three peptides (80, 100 and 110 kDa) predominate (Fig. 1B). In chromatography subsequent to the immunoaffinity procedure (TSK-butyl and Mono S) the 100 and 110 kDa peptides are associated with ß-mannosidase activity while the majority of the 80 kDa peptide (> 50%) elutes prior to the elution of activity (data not shown). The 80 kDa peptide is abundant in the Con A-Sepharose pool and only a very small percent of the peptide (< 10%) is retained by the immunoaffinity column, as judged by Western analysis with anti-80 rabbit serum (data not shown).

Carbohydrate Composition of ß-Mannosidase-To assess the amount and type of N-linked carbohydrate attached to the purified bovine peptides, the TSK pool was digested with Endoglycosidase H and N-Glycosidase F (Fig. 2). N-Glycosidase F, which removes all N-linked oligosaccharides from glycoproteins, reduced the molecular size of the 100 and 110 kDa bovine peptides to 86 and 91 kDa (Fig. 2 lane 1). Endoglycosidase H, which removes the high mannose N-linked oligosaccharides from glycoproteins, reduced the molecular size of these peptides to 98 and 108 kDa (Fig. 2 lane 3).

Analytical Scale Purification of ß-Mannosidase from Normal and Affected Tissues-To characterize the genetic defect in calves affected with ß-mannosidosis, kidney tissue from affected and control animals was subjected to the first two steps of the purification procedure. Equivalent amounts of age-matched normal and affected kidney tissue (60 g each) were

homogenized, bound and eluted from Con A-Sepharose. The pools of glycoproteins recovered from this step (normal and affected) contained almost identical levels of protein and α-mannosidase activity. The glycoprotein pools from the Con A-Sepharose step (normal and affected) were subjected to immunoaffinity purification using two analytical sized columns (0.5 ml of resin). Following each elution step, equal volumes of normal and affected eluates were analyzed by SDS-PAGE. Western analysis (using anti-80 rabbit serum) of the Con A-Sepharose pool from affected tissue indicates that the 80 kDa peptide is absent (Fig. 3, panel A, lane D). Western analysis (using anti-100/110 rabbit serum) of the Con A-Sepharose (panel B) and immunoaffinity purified (panel C) pools from affected tissue indicates that the 100 and 110 kDa peptides are absent (Fig. 3: panel B, lane D and panel C, lane D respectively). Silver staining of the immunoaffinity purified pool from affected tissue (Fig. 3, panel D, lane D) also indicates that the 80, 100 and 110 kDa peptides are absent. To demonstrate that equivalent amounts of lysosomal contents were present in the Con A-Sepharose pools (normal and affected), one blot was probed with anti-ß-hexosaminidase (alpha chain specific) rabbit serum (Fig 3, panel E).

Microsequencing-Microsequencing of the N-terminus of the 100 and 110 kDa bovine peptides (immobilized on Immobilon P) failed to produce significant signals when subjected to automated sequence analysis. To obtain internal protein

sequence information from the 100 kDa peptide, 2 mg of Mono S pool was deglycosylated, S-carboxamidomethylated and then subjected to preparative SDS-PAGE. The alkylated and deglycosylated sample contained three prominent peptides (Fig. 4, panel A). The 86 kDa peptide (100 kDa prior to deglycosylation) was electroeluted from the gel (Fig. 4, panel B), passed through Extracti-gel (Pierce) to remove SDS, lyophilized to dryness, dissolved in 70% formic acid and then subjected to cyanogen bromide cleavage. The resulting peptides were separated by reverse phase chromatography (Fig. 5). When subjected to automated sequence analysis, peak 1 (Fig. 5) yielded 6 residues of sequence and peak 2 (Fig. 5) yielded 9 residues of sequence from two different N-termini (Table II).

## DISCUSSION

Recently we reported that immunoaffinity purified lysosomal ß-mannosidase from goat kidney contains three prominent peptides (80, 90 and 100 kDa) (Sopher et al., 1992). Chromatography subsequent to the immunoaffinity procedure indicated that the 90 and 100 kDa peptides were each associated with ß-mannosidase activity while the 80 kDa peptide was not. We also reported that the 90 and 100 kDa peptides were absent or modified in tissues from goats with ß-mannosidase deficiency while the size and abundance of the

80 kDa peptide was normal. Based upon these observations it appeared that the 80 kDa peptide represented the major contaminant following immunoaffinity chromatography.

In this section I will compare and contrast the purified bovine ß-mannosidase enzyme with the analogous caprine enzyme. Bovine ß-mannosidase was purified 160,000 fold to a specific activity of 2,000 units/mg of protein with a 24% yield. When assessed by Coomassie blue stained SDS-PAGE three peptides (80, 100 and 110 kDa) predominate. Two of these peptides (100 and 110 kDa) are consistently associated with ß-mannosidase activity in chromatography subsequent to the immunoaffinity procedure. Based on Western analysis (using anti-80 as probe) the majority of the 80 kDa peptide in the Con A-Sepharose pool (>90%) does not bind to the immunoaffinity column. The majority of the 80 kDa peptide that does bind to the immunoaffinity column is removed by subsequent chromatography.

Immunoblot analysis, using polyclonal antiserum raised against the 100 kDa bovine peptide (anti-100/110), indicates that the 100 and 110 kDa bovine peptides are immunologically related to the 90 and 100 kDa goat peptides (data not shown). Removal of high mannose oligosaccharides from the bovine peptides with Endoglycosidase H reduced the size of the these peptides (100/110 kDa) to 98 and 108 kDa. Complete deglycosylation with N-Glycosidase F reduced the size of the bovine peptides to 86 and 91 kDa.

Previously we reported that deglycosylation of the

analogous goat peptides (90/100 kDa) with Endoglycosidase H and N-Glycosidase F reduced the size of these peptides to 86/96 kDa and 86/91 kDa respectively (Sopher et al., 1992). This suggests that the bovine and goat peptides contain similar amounts of high mannose carbohydrate (approximately 2-4 kDa as judged by SDS-PAGE) and indicates that the 10 kDa increase in size between the bovine and the analogous goat peptides is largely due to the presence of additional complex carbohydrate on the bovine peptides.

When extracts of kidney tissue from calves with ß-mannosidase deficiency are subjected to Con A-Sepharose and immunoaffinity chromatography, the 100 and 110 kDa peptides are not detected in either the Con A-Sepharose or immunoaffinity pools, as judged by silver staining and Western analysis. This implies that these peptides are either absent in affected kidney tissue or they are structurally altered in such a way that Concanavalin A or the polyclonal and monoclonal antibodies do not bind to them. These findings are consistent with those reported for goats with ß-mannosidase deficiency (Sopher et al., 1992).

The 80 kDa peptide was also not detected in ß-mannosidase deficient bovine kidney tissue. This finding differs from that observed for goats with ß-mannosidosis. Co-workers and I previously reported that the 80 kDa peptide was present in normal amounts in kidney tissue from affected goats (Sopher et al., 1992). The reason for this unexpected variance between

affected goat and bovine tissues with respect to the 80 kDa peptide is unclear. The similarity of this disease in goats and calves at the clinical, biochemical and pathological levels makes this finding all the more intriguing.

Bovine lysosomal ß-mannosidase has been purified. The enzyme preparation contains three prominent peptides (80, 100 and 110 kDa). Two of these peptides (100 and 110 kDa) represent more extensively glycosylated forms of the analogous goat peptides (90 and 100 kDa). The absence or modification of the 100 and 110 kDa peptides in tissues from calves with ß-mannosidase deficiency further substantiates their identity and begins to define the molecular basis of bovine ß-mannosidosis.

Acknowledgments-I would like to thank Christine Traviss, my part time co-worker, for her assistance throughout the duration of this work. I would like to thank Joseph Leykam of the Macromolecular Structure, Sequencing and Synthesis Facility for his advice and assistance with the peptide fragmentation and the reverse phase HPLC.

## REFERENCES

Abbitt, B., Jones, M.Z., Kasari, T.R., Storts, R.W., Templeton, J.W., Holland, P.S. and Castenson, P.E. ß-Mannosidosis in twelve Salers calves. J.Am.Vet.Med.Assoc. 198:109-113, 1991.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. Current protocols in molecular biology, New York: Greene Publishing Associates and Wiley-Interscience (John Wiley & Sons), 1987.

Boyer, P.J., Jones, M.Z., Nachreiner, R.F., Refsal, K.R., Common, R.S., Kelley, J. and Lovell, K.L. Caprine ß-mannosidosis: Abnormal thyroid structure and function in a lysosomal storage disease. Lab.Invest. 63:100-106, 1990a.

Boyer, P.J. and Lovell, K.L. In vitro characterization of oligodendrocytes in caprine ß-mannosidosis. Ann.NY Acad.Sci. 605:424-425, 1990b.

Bryan, L., Schmutz, S., Hodges, S.D. and Snyder, F.F. Bovine ß-mannosidase deficiency. Biochem.Biophys.Res.Comm. 173:491-495, 1990.

Cooper, A., Sardharwalla, I.B. and Roberts, M.M. Human ß-mannosidase deficiency. N.Engl.J.Med. 315:1231, 1986.

Cooper, A., Wraith, J.E., Savage, W.J., Thornley, M. and Noronha, M.J. ß-Mannosidase deficiency in a female infant with epileptic encephalopathy. J.Inherited Metab.Dis. 14:18-22, 1991.

Dorland, L., Duran, M., Hoefnagels, F.E.T., Breg, J.N., Fabery de Jonge, H., van Eeghen-Cransberg, K., van Sprang, F.J. and van Diggelen, O.P. ß-Mannosidosis in two brothers with hearing loss. J.Inherited Metab.Dis. 11:255-258, 1988.

Fontana, A. and Gross, E. Fragmentation of polypeptides by chemical methods. In: Practical protein chemistry: A handbook, edited by A. Darbre, New York: John Wiley and Sons, 1986.

Frei, J.I., Cavanagh, K., Fisher, R.A., Hausinger, R.P., Dupuis, M., Rathke, E.J.S. and Jones, M.Z. Partial purification of goat kidney ß-mannosidase. Biochem.J. 249:871-875, 1988.

Gage, D.A., Rathke, E.J.S., Costello, C.E. and Jones, M.Z. Determination of sequence and linkage of tissue oligosaccharides in caprine ß-mannosidosis by FAB-CAD-MS/MS. Glycoconjugate J. (in press), 1992.

Hartley, W.J. and Blakemore, W.F. Neurovisceral storage and dysmyelinogenesis in neonatal goats. ActaNeuropathol. 25:325-333, 1973.

Healy, P.J., Seaman, J.T., Gardner, I.A. and Sewell, C.A. ß-Mannosidase deficiency in anglo nubian goats. Aust.Vet.J. 57:504-507, 1981.

Hewick, R.M., Hunkapiller, M.W., Hood, L.E. and Dreyer, W.J. A gas-liquid solid phase peptide and protein sequenator. J.Biol.Chem. 256:7990-7997, 1981.

Jacobs, E. and Clad, A. Electroelution of fixed and stained membrane proteins from preparative sodium dodecyl sulfate-polyacrylamide gels in a membrane trap. Anal.Biochem. 154:583-589, 1986.

Jolly, R.D., Thompson, K.G., Bayliss, S.L., Vidler, B.M., Orr, M.B. and Healy, P.J. ß-Mannosidosis in a Salers calf: A new storage disease of cattle. J.New Zealand Vet.Assn. (In Press), 1991.

Jones, M.Z. and Dawson, G. Caprine ß-mannosidosis: Inherited deficiency of ß-mannosidase. J.Biol.Chem. 256:5185-5188, 1981a.

Jones, M.Z. and Laine, R.A. Caprine oligosaccharide storage disease: Accumulation of ß-mannosyl (1-4) ß-N-acetylglucosaminyl (1-4) ß-N-acetylglucosamine J.Biol.Chem. 256:5181-5184, 1981b.

Jones, M.Z., Cunningham, J.G., Dade, A.W., Alessi, D.M., Mostosky, U.V., Vorro, J.R., Benitez, J.T. and Lovell, K.L. Caprine ß-mannosidosis: Clinical and pathological features. J.Neuropath.Exp.Neurol. 42:268-285, 1983.

Jones, M.Z., Rathke, E.J.S., Cavanagh, K. and Hancock, L.W. ß-Mannosidosis: Prenatal biochemical and morphological characteristics. J.Inherited Metab.Dis. 7:80-85, 1984.

Jones, M.Z. ß-Mannosidosis. Comp.Pathol.Bull. 21:2-4, 1989.

Jones, M.Z., Rathke, E.J.S., Gage, D.A., Costello, C.E., Murakami, K., Ohta, M. and Matsuura, F. Oligosaccharides accumulated in the bovine ß-mannosidosis kidney. J.Inherited Metab.Dis. (in press), 1991.

Kleijer, W.J., Hu, P., Thoomes, R., Boer, M., Huijmans, J.G.M., Blom, W., van Diggelen, O.P., Seemanova, E. and Macek, M. ß-Mannoidase deficiency: Heterogeneous manifestations in the first female patient and her brother. J.Inherited

Metab.Dis. 13:867-872, 1990.

Kyosaka, S., Murata, S., Nakamura, F. and Tanaka, M. Purification and kinetic properties of guinea pig liver ß-mannosidase. Chem.Pharm.Bull. 33:256-263, 1985.

Lovell, K.L. and Jones, M.Z. Distribution of central nervous system lesions in ß-mannosidosis. ActaNeuropathol. 62:121-126, 1983.

Lovell, K.L., Jones, M.Z., Patterson, J., Abbitt, B. and Castenson, P. Thyroid structure and function in bovine ß-mannosidosis. J.Inherited Metab.Dis. 14:228-230, 1991.

Matsuura, F., Laine, R.A. and Jones, M.Z. Oligosaccharides accumulated in the kidney of a goat with ß-mannosidosis: Mass spectrometry of intact permethylated derivatives. Arch.Biochem.Biophys. 211:485-493, 1981.

Matsuura, F., Jones, M.Z. and Frazier, S.E. Structural analysis of the major caprine ß-mannosidosis urinary oligosaccharides. Biochim.Biophys.Acta 759:67-73, 1983.

Patterson, J.S., Jones, M.Z., Lovell, K.L. and Abbitt, B. Neuropathology of bovine ß-mannosidosis. J.Neuropathol.Exp.Neurol. 50:538-545, 1991.

Pearce, R.D., Callahan, J.W., Little, P.B., Armstrong, D.T., Kiehm, D. and Clarke, J.T.R. Properties and prenatal ontogeny of ß-mannosidase in selected goat tissues. Biochem.J. 243:603-609, 1987.

Render, J.A., Lovell, K.L. and Keller, C.B. The ocular and otic pathology of bovine ß-mannosidosis. J.Vet.Diag.Invest (in press), 1992.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. Measurement of protein using bicinchoninic acid. Anal.Biochem. 150:76-85, 1985.

Sopher, B.S., Traviss, C.E., Cavanagh, K.T., Jones, M.Z. and Friderici, K.H. Purification and characterization of bovine ß-mannosidase using monoclonal and polyclonal antibodies. J.Biol.Chem. (in press), 1992.

Tjoa, S., Wenger, D.A. and Fennessey, P.V. Quantitative analysis of disaccharides in the urine of ß-mannosidosis patients. J.Inherited Metab.Dis. 13:187-194, 1990.

Tsai, S.F., Bishop, D.F. and Desnick, R.J. Human uroporphyrinogen III synthase: Molecular cloning, nucleotide

sequence and expression of a full-length cDNA. Proc.Natl.Acad.Sci.U.S.A. 55:7049-7053, 1988.

van Pelt, J., Hokke, C.H., Dorland, L., Duran, M., Kamerling, J.P. and Vliegenthart, J.F.G. Accumulation of mannosyl-ß(1-4)-N-acetylglucosamine in fibroblasts and leukocytes of patients with a deficiency of ß-mannosidase. Clin.Chim.Acta. 187:55-60, 1990.

Wenger, D.A., Sujansky, E., Fennessey, P.V. and Thompson, J.N. Human ß-mannosidase deficiency. N.Engl.J.Med. 315:1201-1205, 1986.

TABLE I

Summary of the purification of ß-mannosidase from normal cow kidney

| Purification step | Total activity | Total protein | Specific activity | Yield | Purification |
|---|---|---|---|---|---|
| | units | mg | units/ mg protein | % | -fold |
| High speed supernatant | 18,700 | 1,460,000 | 0.0128 | 100 | 1 |
| Con A-Sepharose chromatography | 10,000 | 6,050 | 1.65 | 53 | 129 |
| Immunoaffinity chromatography | 6,000 | 7.0 | 860 | 32 | 67,000 |
| TSK-butyl chromatography | 4,700 | 3.3 | 1,400 | 25 | 110,000 |
| HPLC: Mono S chromatography | 4,500 | 2.3 | 2,000 | 24 | 160,000 |

Similar results were obtained in a second experiment.

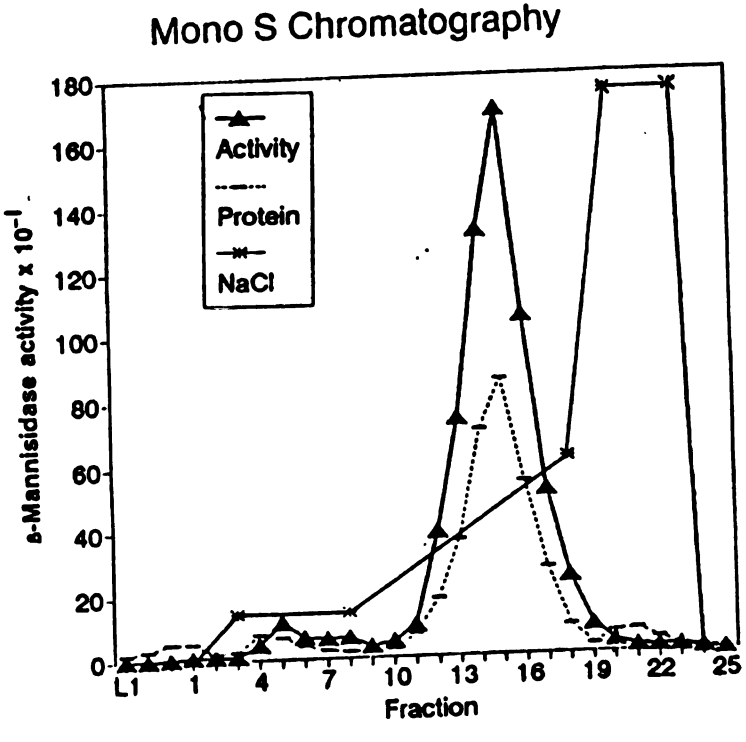
TABLE II

Summary of the protein sequence results

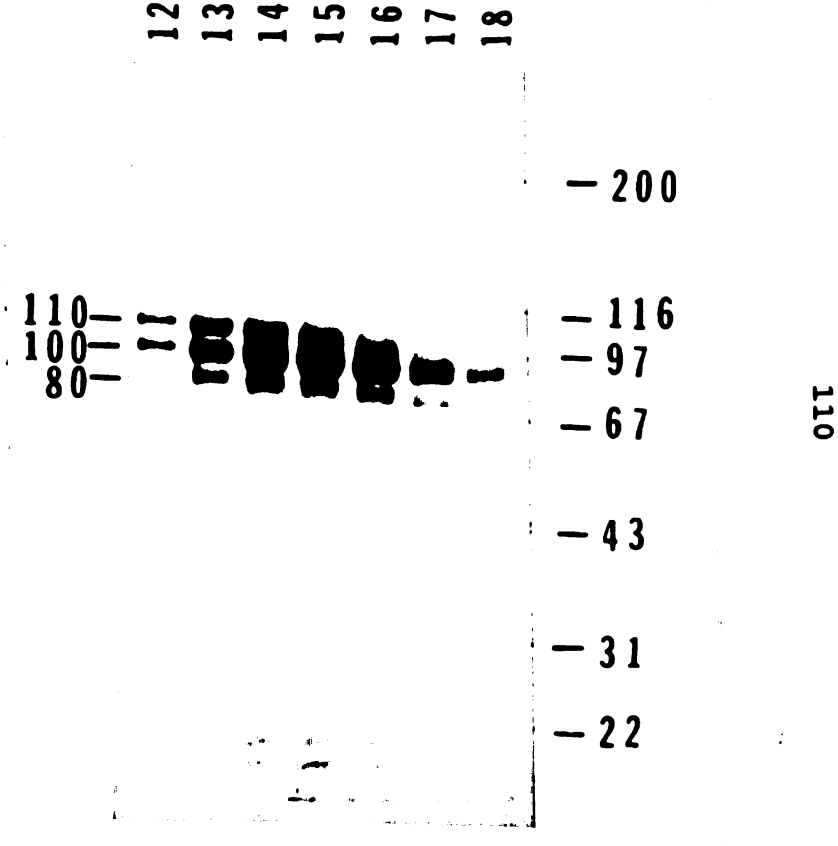
| Protein fractions (Fig. 5) | Cycle # | Residue(s) |
|---|---|---|
| 1 | 1 | F |
| | 2 | T |
| | 3 | P |
| | 4 | I |
| | 5 | Y |
| | 6 | D |
| 2 | 1 | G/L |
| | 2 | A/Y |
| | 3 | S/P |
| | 4 | D/T |
| | 5 | L |
| | 6 | H/K |
| | 7 | D/S |
| | 8 | F |
| | 9 | Q/E |

Fig. 1. **Cation-exchange chromatography of bovine ß-mannosidase.** ß-Mannosidase activity from the TSK-butyl procedure was pooled and dialyzed into Mono-S buffer. The dialyzed sample was subjected to Mono-S chromatography (panel A). The enzyme was eluted from the Mono-S column with a multi-step NaCl gradient (0-1 M NaCl). Protein was monitored at $A_{280}$. Aliquots (25 uL) from selected 1 mL fractions were subjected to SDS-PAGE analysis (panel B). Protein bands were visualized by Coomassie blue staining. The position of molecular weight standards (in kDa) is indicated on the right of panel B.

Fig. 1


Mono S Chromatography
Activity
Protein
NaCl
α-Mannisidase activity x 10⁻¹
Fraction
L1
1
4
7
10
13
16
19
22
25
0
20
40
60
80
100
120
140
160
180

A


12
13
14
15
16
17
18
110
100
80
200
116
97
67
43
31
22

B

110

Fig. 2. **Glycosylation of affinity purified ß-mannosidase.** Immunoaffinity purified ß-mannosidase (2 ug) was deglycosylated with N-Glycosidase F (lane 1), no glycosidase (lane 2) or Endoglycosidase H (lane 3). The digestion mixtures were resolved on SDS-PAGE (7.5% acrylamide) and protein bands were visualized by silver staining. The position of molecular weight standards (in kDa) is indicated on the right of the figure.

Fig. 2

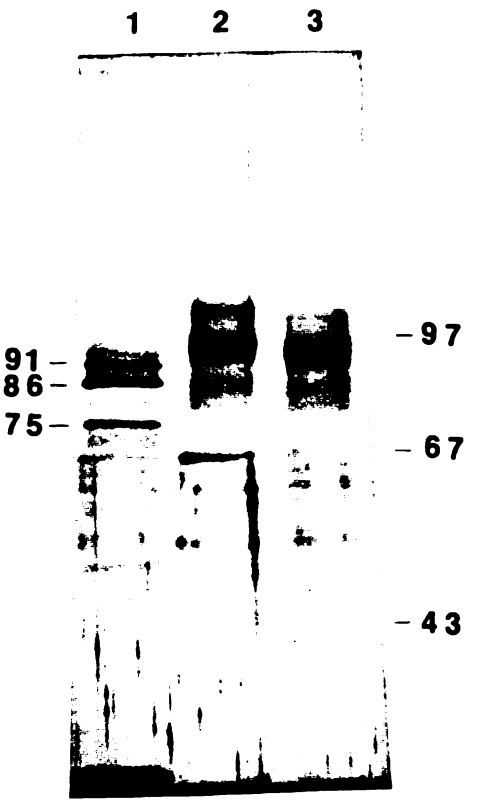


Fig. 3. **Comparative analysis of ß-mannosidase purified from normal and affected goat tissues.** Kidney tissue from normal and affected calves was subjected to Con A-Sepharose and immunoaffinity chromatography. In panels A, B and E equal volumes of the Con A-Sepharose pools from normal (N) and ß-mannosidase deficient (D) tissue were subjected to Western analysis. Panel A was probed with anti-80 rabbit serum, panel B was probed with anti-100/110 rabbit serum and panel E was probed with anti-ß-hexosaminidase (α-chain specific) rabbit serum. In panels C and D equal volumes of the immunoaffinity pools from normal (N) and ß-mannosidase deficient (D) tissue were subjected to SDS-PAGE and Western analysis. Panel C is a Western blot probed with anti-100/110 rabbit serum and panel D is an acrylamide gel stained with silver. The position of molecular weight standards (in kDa) is indicated on the right of the panels.

Fig. 3

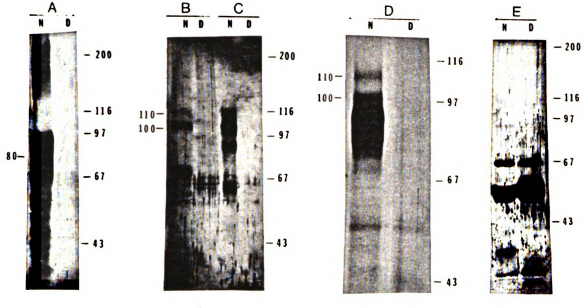

A
N D
200
116
97
80
67
43
B
C
N D
N D
110
100
200
116
97
67
43
D
N D
110
100
116
97
67
43
E
N D
200
116
97
67
43

Fig. 4. **SDS-PAGE analysis of S-carboxamidomethylated and deglycosylated bovine ß-mannosidase prior to and following electroelution of the 86 kDa peptide.** The Mono-S pool (2 mg) was deglycosylated and cysteines were alkylated with iodoacetamide. Panel A: S-carboxamidomethylated and deglycosylated bovine ß-mannosidase (6 ug) was subjected to SDS-PAGE and protein bands were visualized by Coomassie blue staining. The deglycosylated and S-carboxamidomethylated sample (approximately 1 mg) was subjected to preparative SDS-PAGE. A gel slice containing the 86 kDa peptide (100 kDa prior to deglycosylation) was excised and the protein was electroeluted from the acrylamide. Panel B: Following electroelution a fraction of the total electroeluted pool (0.5%, approximately 2 ug) was subjected to SDS-PAGE and protein was visualized by Coomassie blue staining. The position of molecular weight standards (in kDa) is indicated on the right of the panels.

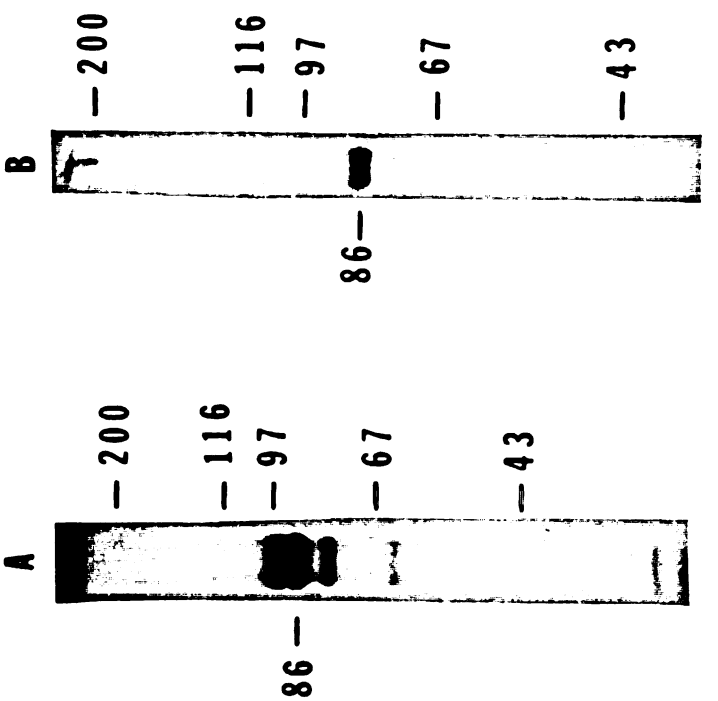



Fig. 4

Fig. 5. **Fractionation of cyanogen bromide fragments of the electroeluted 86 kDa bovine peptide.** The separation was achieved on an Aquapore RP-300 column (C8, 1X250 mm) using 0.1% trifluoroacetic acid with a linear 60 minute gradient from 0 to 90% acetonitrile.

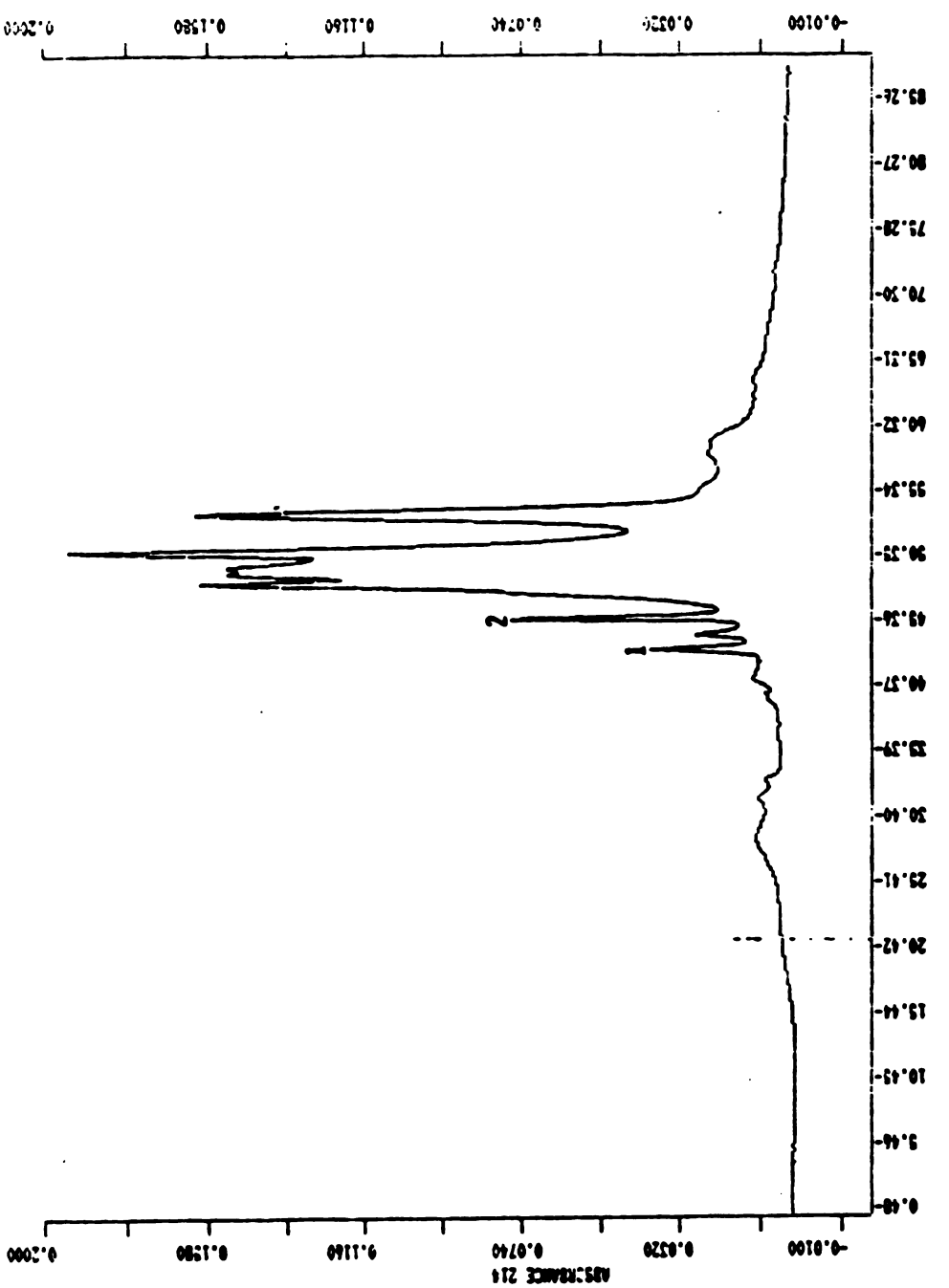



Fig. 5

# Concluding Remarks

Goat and bovine ß-mannosidases can now be reproducibly purified 100,000 to 200,000 fold in relatively high yield (approximately 25%). In both species the enzyme consists of two related peptides which both appear to be associated with ß-mannosidase activity. A third peptide (80 kDa) which is not associated with ß-mannosidase activity in vitro is absent in tissues from ß-mannosidase deficient calves but not goats.

Purification of the ß-mannosidase enzyme has led to the production of specific polyclonal antisera which are being used to screen cDNA expression libraries. The purified enzyme has also yielded some "limited" peptide sequence information and additional protein is currently being prepared for further sequence analysis. This work is expected to lead to the cloning of the ß-mannosidase gene.

Cloning of the ß-mannosidase gene will facilitate the characterization of the processing of this enzyme. Understanding how this enzyme is transported and processed may lead to insights regarding the nature of the relationship between the ß-mannosidase peptides and possibly their relationship to the 80 kDa peptide (if a relationship exists).

Cloning of this gene will make possible the molecular characterization of the normal gene and the ß-mannosidosis mutations. Comparisons of the genes and associated disease mutations in humans, goats and cattle will facilitate the structural characterization of the ß-mannosidase enzyme. Understanding the structure and associated function(s) of ß-

mannosidase could possibly lead to insights regarding the molecular basis of the myelin deficits in ruminants, the clinical heterogeneity in humans, and possibly the unexpected absence of the 80 kDa peptide in ß-mannosidase deficient bovine tissue.

Cloning of the ß-mannosidase gene would make caprine ß-mannosidosis a disease suitable for the development and evaluation of gene therapy approaches. The severe and consistent phenotypic expression of ß-mannosidosis in goats, which is unusual for a lysosomal storage disease, make this disease exceptionally well suited for the evaluation of prenatal therapeutic interventions.