Comparative Characterization Of Rodent And Human Alpha-l-fucosidases.

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Comparative characterization of rodent and human α-L-fucosidases

Shoarinejad, Fariba, Ph.D.

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COMPARATIVE CHARACTERIZATION OF RODENT AND HUMAN α-L-FUCOSIDASES

by

Fariba Shoarinejad

A Dissertation

Presented to the Graduate Committee

of Lehigh University

in Candidacy for the Degree of

Doctor of Philosophy

in

Chemistry

Lehigh University

March 1994

Dedication

This dissertation is dedicated

to my parents,

Ali Akbar Shoarinejad and Fatemeh Alizadeh Ashrafi

who gave me encouragement

to my childern,

Hadi, Sahar, and Nassim

for making it all worthwhile

and

to my husband

Mehdi Durali

who has given me his patience,

understanding, and love through it all.

Certificate of Approval

Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dr. Jack A. Alhadeff, Advisor

Jack A. Alhadeff

Special committee directing
the doctoral work of
Fariba Shoarinejad

Dr. Michael J. Behe

Michael J. Behe

Dr. Keith J. Schray

Keith J. Schray

Dr. Vassie C. Ware

Vassie C. Ware

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List of Abbreviations Used

General Abbreviations

A_{280}	absorbance at 280 nanometers
Approx.	approximately
BCIP	5-bromo-4-chloro-3-indolyl phosphate
β-ME	β-mercaptoethanol
Da	Dalton
DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
4-MU	4-methylumbelliferyl-α-L-fucopyranoside
Gal	galactose
HSA	human serum albumin
IEF	isoelectric focusing
IgG	Immunoglobulin G
kbp	kilo base pair
kDa	kilodaltons
mA	milliampere
MAb	monoclonal antibody
Man	mannose
M_r	molecular weight
NBT	Nitro Blue Tetrazolium

NP-40	nonidet p-40
PAb	polyclonal antibody
pI	isoelectric point
PNP-Fuc	p-nitrophenyl-α-L-fucopyranoside
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
U/mg	units per milligram
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

Amino Acid Abbreviations

Arg	Arginine
Asp	Aspartic acid
Leu	Leucine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine

ABSTRACT

α-L-fucosidase is a lysosomal hydrolase which is involved in the hydrolytic degradation of structurally diverse fucoglycoconjugates. Preliminary investigations have suggested that mouse liver α-L-fucosidase is different from other mammalian fucosidases studied to date because of the fact that isoforms with pI values above 7.0 comprise approximately 25% of the total activity of the mouse enzyme. In addition, comparison of liver α-L-fucosidase from several rodents (mouse, guinea-pig, rat, and hamster) indicated that only the mouse and hamster liver α-L-fucosidases contain basic isoforms. Isoelectric focusing of brain and spleen α-L-fucosidase from four rodents (mouse, guinea-pig, rat, and hamster) indicated that only mouse and hamster tissues contained isoforms with significant amounts of activity above pI 7.0.

Mouse liver α-L-fucosidase was purified 18,700-fold by immunoaffinity chromatography using polyclonal (PAb) goat-anti-human liver α-L-fucosidase IgG coupled to Sepharose 4B. The final specific activity was 7500 U/mg protein, and a 66% yield was achieved. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated the presence of two major protein bands (57 and 62 kDa)for mouse fucosidase. Western blotting indicated that both bands were immunoreactive with polyclonal antibodies (PAbs) and/or monoclonal antibodies (MAbs) raised against human liver fucosidase. The lectins, SNA

and GNA, recognized the two mouse protein bands, indicating that both subunits are glycosylated and contain sialic acid residues.

Hamster liver α-L-fucosidase was purified approximately 57,000-fold with a yield of 80% by affinity chromatography on agarose-ϵ-aminocaproyl-fucosamine. The final specific activity was 24,700 nmol/min/mg protein. SDS-PAGE analysis of hamster liver α-L-fucosidase indicated the presence of one to two closely-spaced subunits at 56 and 60 kDa. Dot immunoblotting and Western blotting analysis indicated the hamster enzyme was recognized by polyclonal antibodies but not by a monoclonal antibody (both antibodies were prepared against human liver α-L-fucosidase). The pH-activity curve of purified hamster liver α-L-fucosidase was broad with an optimum centered around pH 6.8, and with high activity between pH 5.5 and 7.5.

The subunits of human liver α-L-fucosidase were separated by SDS-PAGE, excised, and subjected to peptide mapping after CNBr cleavage. The CNBr peptide maps of the glycosylated 56 and 51 kDa subunits were similar, except that several peptides from the larger subunit were shifted to higher apparent molecular weights when compared to their counterparts in the smaller subunit. These molecular weight differences were almost completely eliminated when CNBr-peptide mapping was performed on the deglycosylated 48 and 45 kDa polypeptides, suggesting that the molecular weight differences were due to carbohydrate differences. Minor differences not related to

glycosylation were found in the CNBr peptide maps of the 48 and 45 kDa polypeptides. Three of the 16 peptides found in the larger polypeptide were not found in the smaller polypeptide.

Sequence analysis suggested that both the 48 and 45 kDa polypeptides were blocked at their amino-termini, but analysis of the largest CNBr peptide from each polypeptide indicated an identical 13-amino acid sequence near the amino-termini.

The overall results indicated that the subunits of human liver fucosidase are very similar but minor differences exist. These are probably due to post-translational modifications including, but not limited to, glycosylation.

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Lysosomal hydrolases are an important class of enzymes which catalyze the hydrolysis of macromolecules into their components (e.g. amino acids, fatty acids, monosaccharides). These enzymes are assembled as precursor proteins on ribosomes associated with the endoplasmic reticulum (ER). Following co-translational and post-translational modification in the ER and Golgi apparatus, lysosomal enzymes are primarily sequestered into lysosomes via a receptor which is specific for a phosphorylated mannose residue, a feature common to this group of enzymes [1].

α-L-Fucosidase (α-L-fucoside fucohydrolase, E.C. 3.2.1.51) is a lysosomal glycosidase which catalyzes the hydrolysis of L-fucose from a diverse and widespread group of hydrophilic and hydrophobic fucose-containing glycoconjugates (Table 1.1) [from ref. 2]. These molecules contain L-fucose in various α-glycosidic linkages (α1-2, α1-3, α1-4 and α1-6) primarily to galactose, and N-acetylglucosamine [3], and are synthesized by at least five different fucosyl-transferases [4]. The methyl pentose, L-fucose, is a common constituent of many biological molecules including oligosaccharides, glycolipids and glycoproteins. For example, human milk oligosaccharides (2'- and 3'-fucosyllactose, lacto-N-fucopentose I, II, and III), blood group substance (H, A, B, Le^a, Le^b), immunoglubins (IgG, IgM, IgA), serum glycoproteins (α_2-macroglobulin, ceruloplasmin,

Table 1.1: Substrate specificity of purified mammalian α-L-fucosidase [from ref. 2]

α-L-fucosidase source	Substrate(s)	Linkage(s) hydrolyzed
Human liver	Milk oligosaccharides	Fucα(1-2)Gal
	Blood group H oligosacch.	Fucα(1-2)Gal
		Fucα(1-4)GlcNac
	Ganglioside Fuc-G_{M1}	Fucα(1-2)Gal
	Ganglioside Fuc-G_{M1}, Fuc-G_{D1b}	Fucα(1-2)Gal
Human brain	Ganglioside Fuc-G_{M1}	Fucα(1-2)Gal
	Oligosaccharide	Fucα(1-2)Gal
Human serum	Synthetic di, trisaccharides	Fucα(1-2)Gal
	and aryl disaccharides	Fucα(1-3)GlcNAc
		Fucα(1-4)GlcNAc
		Fucα(1-6)GlcNAc
	Oligosaccharides	Fucα(1-2)Gal
		Fucα(1-3)GlcNAc
		Fucα(1-4)GlcNAc
		Fucα(1-6)GlcNAc
		Fucα(1-6)GlcNAc
	Bromelain (glycoprotein)	
Human spleen	Milk oligosaccharides	Fucα(1-2)Gal
		Fucα(1-4)GlcNAc
	Oligosaccharides	Fucα(1-2)Gal
		Fucα(1-3)GlcNAc
		Fucα(1-4)GlcNAc
		Fucα(1-6)GlcNAc
	H antigen glycolipid	Fucα(1-2)Gal
Human fibroblasts	Oligosaccharides	Fucα(1-2)Gal
		Fucα(1-3)GlcNAc
		Fucα(1-4)GlcNAc
		Fucα(1-6)GlcNAc
Human fibroblasts	Glycopeptide	Fucα(1-6)GlcNAc
Rat liver	Milk oligosaccharides	Fucα(1-2)Gal
		Fucα(1-3)GlcNAc
		Fucα(1-4)GlcNAc
	β-Glucuronidase glycopeptide	
Rat epididymis	Glycopeptides	Yes
	Glycoprotein	No
Porcine kidney	Fragments of blood group A and H substances	Fucα(1-2)Gal
	Milk oligosaccharides	Fucα(1-2)Gal
		Fucα(1-3)GlcNAc
		Fucα(1-4)GlcNAc
Porcine thyroid	Milk oligosaccharides	Fucα(1-2)Gal
	Submaxillary mucin trisacch.	
	Thyroglobulin	
Canine liver	Fucosidosis storage products	Fucα(1-3)GlcNAc
		Fucα(1-6)GlcNAc
Mouse liver	Milk oligosaccharides	Fucα(1-2)Gal
		Fucα(1-3)GlcNAc
		Fucα(1-4)GlcNAc
	Ganglioside Fuc-G_{D1b}	Fucα(1-2)Gal
	Ganglioside Fuc-G_{M1}	No

orosomucoid), several hormones (thyroglobulin, chorionic gonadotropin) and enzymes (ribonuclease, α-amylase, lactose synthase), mucous secretions (gastric juice, ovarian cyst proteins, submaxillary mucins), and mucopolysaccharides (cartilage and corneal keratin sulfate) have all been shown to contain L-fucose [3]. This partial list of fucose-containing macromolecules points to the importance of α-L-fucosidase in mammalian metabolism. It is known that both the biosynthesis and degradation of the carbohydrate portions of these molecules proceeds in a step-by-step, sequential manner [3]. Therefore, if α-L-fucosidase is deficient or absent, the terminal L-fucose cannot be hydrolyzed. Given the step-by-step nature of the degradative process, loss of α-L-fucosidase activity accounts for accumulation of fucose-containing molecules in the various tissues in which they are found.

A deficiency in α-L-fucosidase activity results in the fatal neurovisceral storage disease, fucosidosis. This disorder, first described by Durand et al. [5] in 1966, is hallmarked by an absence or decrease in α-L-fucosidase activity and an accumulation of fucoglycoconjugates in the brain and visceral tissues of afflicted individuals.

α-L-Fucosidase is ubiquitous in nature and has been studied from a wide variety of organisms including bacteria [6-8], protozoa [9], molds [10,11], fungi [12-14] and mammals [3]. Mammalian α-L-fucosidases have been studied from numerous tissues, cells and fluids including: human brain [15-17],

kidney [18], spleen [19], placenta [20-22], liver [23-25], epididymis [26], leucocytes [27-29], cultured fibroblasts [30], serum [31,32], and amniotic fluid [18,33]; rat brain [34], liver [35], epididymis [36,37], and testis [38]; mouse liver [39,40], spermatazoa [41], teratocarcinoma cells [42] and tissues [43]; monkey brain [44,45]; porcine thyroid [46] and kidney [47,48]; bull seminal plasma and reproductive organs [49,50]; canine liver [51]; rodent livers [52]; and vertebrate livers [53] and serum [54]. These studies have indicated that, in general, mammalian α-L-fucosidases are very similar, if not identical, with respect to various physical and chemical properties including electrophoretic mobility, immunochemical reactivity, and polypeptide composition. Considering the diversity of fucose-containing compounds in nature, however, the existence of another mammalian α-L-fucosidase is certainly possible.

1.2. CHARACTERIZATION STUDIES ON UNPURIFIED α-L-FUCOSIDASE

Studies on unpurified α-L-fucosidase, although providing some valuable information, must be interpreted with caution since the properties of an enzyme in crude supernatants may not reflect its true *in vitro* properties due to the presence of inhibitors, lack of appropriate cofactors, protein-protein interactions, etc. The high specificity of antibodies overcomes some of the problems in working with unpurified enzymes and has led to useful information on unpurified

mammalian α-L-fucosidase [2]. Immunochemical experiments have shown that α-L-fucosidase from human brain, placenta, serum, skin fibroblasts and lymphoid cell lines is recognized by goat anti-human liver α-L-fucosidase IgG (15,22,31,55). Furthermore, α-L-fucosidases from other mammalian livers are recognized by this antibody as well (53). Similarly, monkey brain α-L-fucosidase shows partial immunological identity with non-neural monkey tissue fucosidases in addition to human and rat liver and rat brain α-L-fucosidases (44). Cross-reactivity was also observed between human liver, mouse liver and mouse placental α-L-fucosidases in double-antibody immunoprecipitation experiments (40). These studies suggest that most, if not all, mammalian α-L-fucosidases exhibit some sequence identity and have antigenic sites (epitopes) in common.

Like many other lysosomal hydrolases, α-L-fucosidase has been shown to exist in multiple molecular forms, or isoforms. These forms can be separated by isoelectric focusing (IEF) which allows the resolution of polypeptides differing in their isoelectric points (pIs). Isoelectric focusing of human liver α-L-fucosidase by column and gel yields four to eight isoforms with pI values between 4.0 and 7.5 [56,57]. It has been shown that these forms are related to one another, at least in part, by sialic acid residues [24,56-58]. In addition, these isoforms show subtle differences in certain kinetic properties including thermostability [59], Michaelis constant (Km) and pH

curves [60].

Mouse liver α-L-fucosidase is different from other mammalian fucosidases studied to date because of the presence of unusual basic isoelectric forms (isoforms) (Figure 1.1) [from 40]. These basic isoforms, with approximate pI values of 7.0, 8.3, and 9.0 comprise about 25% of the total α-L-fucosidase activity in the profile, while the remainder of activity is distributed in 3 to 4 isoforms between approximate pI values of 4 and 7 [40]. The unusual properties of mouse α-L-fucosidase have prompted the study of α-L-fucosidase derived from the livers of hamster, guinea-pig and rat to determine whether the properties of α-L-fucosidase are similar in other members of the Order Rodentia [52]. The results indicated that α-L-fucosidase from hamster liver, but not from rat and guinea-pig livers, is similar to the mouse enzyme with respect to its isoform profile, and contains three isoforms with pI values of 8.2, 9.0, and 9.8 (Figure 1.2) which comprise about 50% of the total activity [52]. The relative distributions of α-L-fucosidase activity above and below pI 7.0 for mouse, hamster, rat and guinea-pig are summarized in Table 1.2 [from ref. 52].

1.3. CHARACTERIZATION STUDIES ON PURIFIED α-L-FUCOSIDASE

The purification of various mammalian α-L-fucosidases including human liver [23,24,61], placenta [20-22], spleen [19], serum [31,32], and brain [15,55]; rat liver [35] and epididymis [37,62]; monkey brain [44] and porcine thyroid [46]

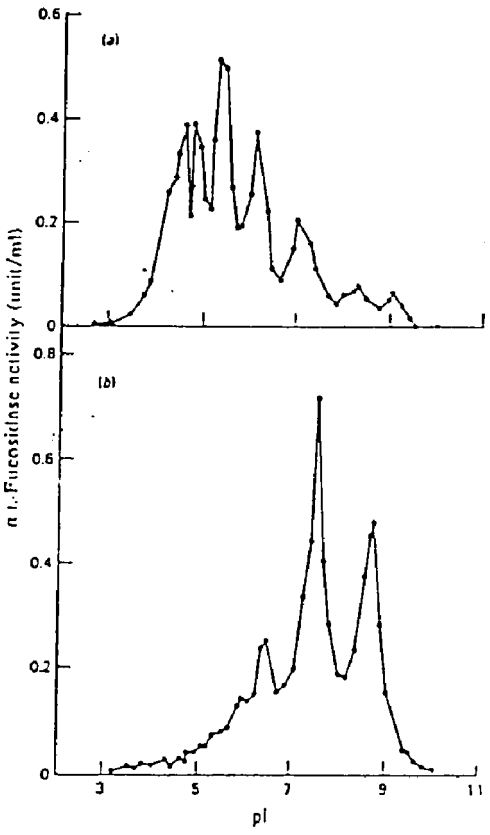


Figure 1.1: Isoelectric focusing profile of α-L-fucosidase from adult mouse liver supernatant fluid (a) and from 12 hr neuraminidase adult mouse liver supernatantfluid (b) [43].

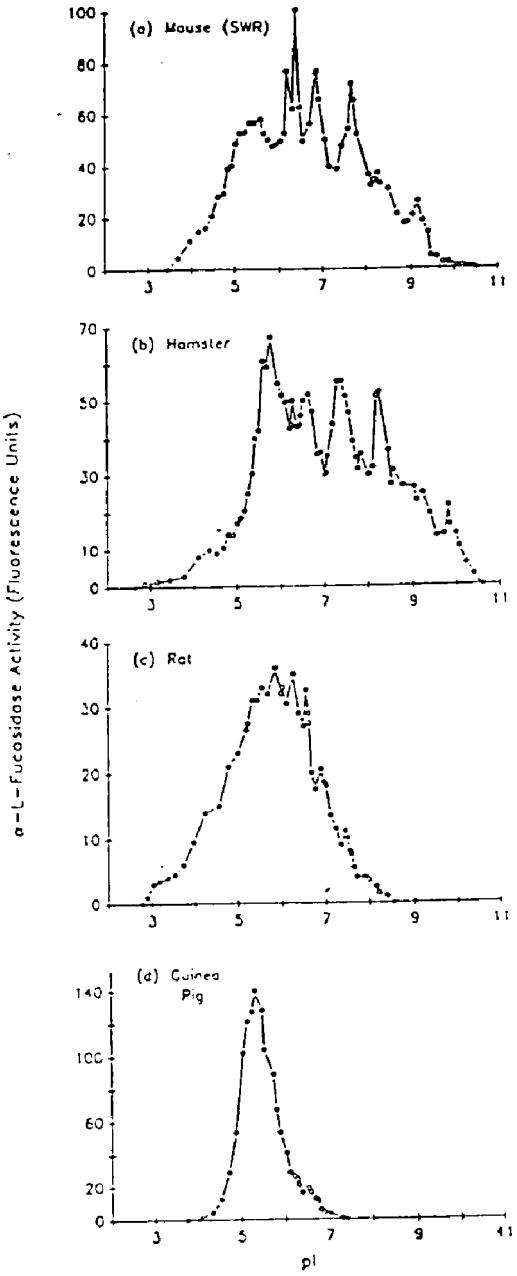


Figure 1.2: Isoelectric focusing profiles of α-L-fucosidase from Swiss Webster mouse (a), hamster (b), rat (c), and guinea-pig (d) liver supernatant fluids [from ref. 52].

Table 1.2: Relative Distribution of Rodent Liver α-L-fucosidase Activity in the Isoelectric-focusing Profiles [from ref. 52].

Species	Strain	Relative Distribution (% of recovered activity)	
		Below pI 7.0	Above pI 7.0
Mouse	Swiss Webster	69	31
Hamster	Syrian	50	50
Rat		93	7
Guinea-pig	Hartley	100	0

has been achieved by substrate-analogue affinity chromatography using agarose-ε-aminocaproylfucosamine. This procedure resulted in a 6600-fold purification of human liver α-L-fucosidase to apparent homogeneity with a 66% overall yield. The isoform composition of the purified α-L-fucosidase is similar to that of the crude enzyme which indicates that representative forms of the enzyme have been purified.

Because of inability to purify appreciable amounts of active mouse liver α-L-fucosidase using the agarose-ε-aminocaproyl-fucosamine affinity column under various conditions, Laury-Kleintop et al. [39] devised a procedure to purify the enzyme by immunoaffinity chromatography using the IgG fraction of goat-anti-human liver α-L-fucosidase [63] covalently coupled to Sepharose 4B. With this method, mouse liver α-L-fucosidase was purified approximately 26,500-fold with a 10% overall yield.

The successful purification of mammalian α-L-fucosidase has enabled more detailed analysis of various structural and kinetic properties. Monosaccharide compositional analysis performed on α-L-fucosidase from human liver [61,64], human placenta [22], human spleen [19], and rat liver [35] has shown that α-L-fucosidases are glycoproteins which contain 7-8%, by weight, carbohydrate. The types and amounts of monosaccharides found (mannose, galactose, glucose, N-acetyllactosamine, sialic acid and fucose) suggest that α-L-fucosidase could contain oligomannoside-type, N-acetyllactosamine-type, or

hybrid-type oligosaccharide side chains (Table 1.3) [from ref. 2]. The exact function(s) of these carbohydrate structures is unknown. However, earlier preliminary studies suggest that the presence and type of glycosylation affect the stability and kinetics of fucosidase [65].

The molecular weights of mammalian α-L-fucosidases range from approximately 190,000 to 230,000 Daltons as determined by sedimentation equilibrium and density gradient ultracentrifugation (Table 1.3) [from ref. 2]. A molecular weight of 230,000 ± 10,000 has been determined for purified human liver α-L-fucosidase. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) usually separates mammalian α-L-fucosidase into one-to-two closely-spaced protein-staining bands with molecular weights of approximately 50-60 KDa. This, in conjunction with the molecular weight data for the whole enzyme, indicates that the enzyme contains four subunits. Some of the major kinetic properties of purified mammalian α-L-fucosidases are summarized in Table 1.4 [from ref. 2]. Mammalian α-L-fucosidases show broad pH-activity curves for the synthetic substrates, P-nitrophenyl-α-L-fucopyranoside (PNP-fucoside), and 4-methylumbelliferyl-α-L-fucopyranoside (4-MU-fucoside), with highest activities between pH values of 4.0 and 6.0. A neutral shoulder is often present in the profile between pH 6 and 7. The pH optimum of human fucosidase extracted from liver

Table 1.3: Structural Properties of Purified Mammalian α-L-Fucosidases [from ref. 2]

α-L-fucosidase source	Whole enzyme M_r (x 1000)	Subunit(s) M_r (x 1000)	Number of isoforms	Presence and/or type of carbohydrate
Human liver	175*	54,59	8	Yes
	230†	-	-	-
	-	51,57	4-5	Man, Glc, Gal GlcNAc, NANA
Human brain	-	51	7	-
	-	54	6	-
Human serum	296*	54,56.3	7	NANA
	390*	63	7	-
Human placenta	50,200*	-	4	-
	305[a]	25,51,55	5	Man, GlcNAc
Human spleen	50,100*	50	-	Man,GlcNAc,NANA
Monkey brain	285*	73.5	6	-
Porcine thyroid	192‡	55	10	NANA
Rat epididymis	210-220†	47,60	1	Yes
	-	47-50	6	-
	-	50,57	-	-
	-	54	-	-
Rat liver	160[c],217†	55	Multiple	Man,Gal,GlcNAc Glc,NANA,Fuc
	300*			
Mouse liver	-	57,63	Multiple	-
Canine	90-150*	45-50	-	-
	50*			

* Determined by gel filtration chromatography.
† Determined by sedimentation equilibrium analysis.
‡ Determined by density gradient ultracentrifugation

Table 1.4: Kinetic Properties of Purified Mammalian α-L-Fucosidases [from ref. 2]

α-L-fucosidase source	V_{max} (μmol/min/mg) PNP*	4-MU†	Michaelis constants (mM) PNP*	4-MU†	pH optima PNP*	4-MU†
Human liver	19.6	14.1	0.43	0.22	4.6(6.5)‡	-
	-	-	1.1	-	-	-
	-	-	-	0.06	-	3.9-4.0(6-7)
Human brain:						
soluble	10.7	-	0.44	-	4.7(6.6)	4.7(6.6)
pellet	-	-	-	0.07	-	5.5
Human serum	-	-	0.52	-	4.8(6.1)	-
	11.6	-	0.18	-	5.0	-
Human placenta	-	-	0.38	0.3	5.5	6.0
Human spleen						
I	-	-	-	0.13	-	5.3
II	-	-	-	0.06	-	5.0
Monkey brain	15.2	-	0.22	-	5.0(6.6)	-
I	-	-	-	-	5.0	-
II	-	-	-	-	4.0	-
Porcine thyroid	-	-	-	-	5.1	-
Rat cerebral cortex	-	-	1.33	-	4.3	-
Rat epididymis	-	-	-	-	6.3	-
	-	-	0.27	-	-	-
Rat liver	27.0	-	0.19	-	5.7-5.9	-
Mouse liver	-	-	-	0.05	-	5.5(4.0)
Canine liver	-	-	-	0.28	-	7(5.5)

* Determined using *p*-nitrophenyl-α-L-fucoside.
† Determined using 4-methylumbelliferyl-α-L-fucopyranoside.
‡ pH of shoulder of activity in parentheses

is centered at a pH value of 4.6 with a shoulder at pH 6.5 for the p-nitrophenyl-α-L-fucoside substrates. Mouse liver α-L-fucosidase has one pH optimum (5.5) and an acidic shoulder around pH 4.0. Hamster liver α-L-fucosidase has an extremely broad pH optimum centered around pH 6.0 with very high activity between pH values of 3.0 and 5.0. Rat liver α-L-fucosidase exhibits one pH optimum (5.7-5.9).

Analysis of the structural gene for α-L-fucosidase has been undertaken to provide additional information complementary to that obtained from studies on the enzyme protein. To date, cloning studies have provided evidence for only one human fucosidase polypeptide [66-68] and a single rat fucosidase polypeptide [69]. The amino acid sequence of human [66-68] and rat [69] liver α-L-fucosidase has been deduced from cDNA clones. Compilation of cDNA sequences resulted in the determination of a nucleotide sequence of 2053 base pairs encoding α-L-fucosidase [68]. Recently, Kretz et al. [70] reported the determination of the human fucosidase gene structure and sequence as well as the sequence of the fucosidase pseudogene. Their results indicated that the gene encoding fucosidase is composed of eight exons spanning 23 kb of DNA. Analysis of the sequence upstream of the open reading frame has revealed several possible transcription factor binding sites. Northern blot analysis confirmed an mRNA size of 2.3 kb in human lymphoblasts, testis, and epithelial cells [70]. Kretz et al. also sequenced the processed pseudogene of

fucosidase. The sequence of the pseudogene was 80% identical to that of fucosidase cDNA. The protein sequence deduced from cDNA consists of a 22 amino acid signal peptide, and 439 amino acids which comprise the mature polypeptide. There are four potential N-linked glycosylation sites per subunit [66,69]. In addition, four Tyr-x-x (x=Tyr, Trp or Phe) sequences, which are common to several lysosomal hydrolases and may play a role in the mannose-6-phosphate targeting of these lysosomal enzymes [71], are present and conserved in human and rat α-L-fucosidases.

The cDNA of a developmentally regulated gene product from cyclic AMP-stimulated *Dictyostelium discoideum* has also been shown to contain approximately 40% sequence identity with human and rat liver α-L-fucosidase [72]. This gene product may be a fucosidase which hydrolyzes fucoglycoconjugates involved in aggregation or later developmental events in *D. discoideum*.

To date, two mutations, that appear to cause fucosidosis have been found. A single base change at the EcoR1 site in the cDNA has been found as the disease-causing mutation in six patients from five fucosidosis families [73,74]. This C to T transition creates a premature, in-frame TAA stop codon that deletes the carboxy-terminal portion of the protein [74]. The second is a deletion of two exons from the 3′ end of the fucosidase gene [75]. This deletion, which results in negligible fucosidase enzyme activity, has been identified in one family. An analysis of the subunit/polypeptide composition

of human α-L-fucosidase should contribute to a better understanding of the molecular genetics of fucosidosis.

Subunit compositional analysis of human α-L-fucosidase has led to variable results with most initial electrophoretic studies showing only one subunit (24,35,46). Circumstantial evidence for one subunit also comes from the fact that only a single cDNA for human α-L-fucosidase has been cloned [66,68]. However, several studies have provided evidence for two non-identical subunits with very closely-related molecular weights of approximately 51 and 56 KDa for fucosidase from human liver [58,61,76], spleen [19], and serum [31]. The confusion as to whether human α-L-fucosidase consists of one, or more subunits appears to result from the fact that SDS-PAGE cannot readily resolve subunits with similar molecular weights (i.e.,±10%), particularly at the high protein loading (>20 μg) used in some studies. In addition, the fact that α-L-fucosidase is a glycoprotein with approximately 7%, by weight, N-glycans [77] adds to the confusion since the two observed bands with similar molecular weights may be related solely by glycosylation differences.

Recent Western blotting studies on human liver α-L-fucosidase have provided evidence for two non-identical subunits by demonstrating that the 56 and 51 KDa polypeptides are recognized differentially by four monoclonal antibodies and by two lectins [78]. Also, two different polypeptides are still present after removal of carbohydrate with N-glycanase

[78]. This latter finding suggests that the two α-L-fucosidase subunits are not related solely by glycosylation differences, but that other post-translational modifications and/or polypeptide differences exist.

The subunit composition of several mammalian α-L-fucosidases has been determined by SDS-PAGE [15,24,31,35,37,39,44,55,79,80,81]. The results indicate that most α-L-fucosidases, with the exception of the monkey brain enzyme, contain at least one subunit with a molecular weight of 50-70 kDa. The idea that various α-L-fucosidases share a common subunit is supported by the immunochemical experiments described earlier in which cross-reactivity was observed between the enzyme from several different tissues and species. The properties of rodent liver α-L-fucosidase, particularly the anomalies of the mouse enzyme and its unique isoelectric focusing profile described thus far, have prompted further studies on other tissues from four rodents. Chapter Two of this dissertation will focus on analysis of brain and spleen tissues from mouse, guinea-pig, rat, and hamster to determine if they contain the unusual fucosidase isoforms above pI 7.0. Chapter Three will employ immunological analysis to study the structural relatedness of the subunits of mouse liver α-L-fucosidase and the results will be compared to the results of similar studies on human liver α-L-fucosidase [50]. Chapter Four will describe characterization studies on purified hamster liver α-L-fucosidase with regard to its isoform

profile, pH optimum and subunit composition. In addition, the enzyme's antigenic relatedness to mouse and human liver α-L-fucosidase will be investigated.

Western-blot analysis of human liver α-L-fucosidase indicates that variable N-glycosylation contributes to, but does not completely account for, structural differences in the subunits of fucosidase [50]. Chapter Five will describe the results of N-terminal and peptide mapping studies to determine whether primary amino acid sequence alterations and/or post-translational modifications contribute to the observed subunit differences of fucosidase. And finally, chapter Six summarizes the dissertation with recommended extensions of the current work.

1.4. REFERENCES:

1. Dahms, N.M., Lobel, P. and Kornfeld, S. (1989) *J. Biol. Chem.* **264**, 12115-12118.

2. Johnson, S.W., and Alhadeff, J.A. (1991) *Comp. Biochem. Physiol.* **99B**, 479-488.

3. Alhadeff, J.A. and O'Brien, J.S. (1977) in *Practical Enzymology of the Sphingolipidoses* (Glew, R.H. and Peters, S.P., eds.) pp. 247-281, Alan R. Liss, New York.

4. Beyer, T.A., Sadler, J.E., Rearick, J.I., Paulson, J.C., and Hill, R.L. (1981) *Adv. Enzymol.* **52**, 23-175.

5. Durand, P., Borrone, C. and Della Cella, G. (1966) *Lancet* **2**, 1313-1314.

6. Mortensson-Egnund, K., Schoyen, R., Howe, C., Lee, L.T., and Harboe, A. (1969) *J. Bacteriol.* **98**, 924-929.

7. Aminoff, D., and Furukawa, K. (1970) *J. Biol. Chem.* **245**, 1659-1669.

8. Tsuji, Y., Yamamoto, K., and Tochikura, T. (1990) *J. Biol. Chem.* **108**, 235-240

9. Stealey, J.R., Watkins, W.M. (1971) *Biochem. J.* **126**, 16-17.

10. Bahl, O.P. (1970) *J. Biol. Chem.* **745**, 299-304.

11. Yazawa, S., Madiyalakan, R., Chawda, R.P., and Matta, K.L. (1986) *Biochem. Biophys. Res. Commun.* **136**, 563-569.

12. Yano, T., Yamamoto, K., Kumagai, H., Tochikura, T., Yokoyama, T., Seno, T., and Yamaguchi, H. (1985) *Agric. Biol. Chem.* **49**, 3179-3187.

13. Yamamoto, K., Tsuji, Y., Kumagai, H., and Tochikura, T. (1986) *Agric. Biol. Chem.* **50**, 1689-1695.

14. Tsuji, Y., Yamamoto, K., and Tochikura, T. (1990) *Appl. Environ. Microbiol.* **56**, 928-933.

15. Alhadeff, J.A. and Janowsky, A.J. (1977) *J. Neurochem.* **28**, 423-427.

16. Dawson, G. and Tsay. G. (1977) *Arch. Biochem. Biophys.* **184**, 12-23.

17. Hopfer, R.L. and Alhadeff, J.A. (1985) *Biochem. J.* **229**, 679-685.

18. Wiederschain, G.Y., Kolibaba, L.G. and Rosenfeld, E.L. (1973) *Clin. Chim. Acta* **46**, 305-310.

19. Chien, S.F., and Dawson, G. (1980) *Biochim. Biophys Acta* **614**, 476-488.

20. Alhadeff, J.A., Miller, A.L., and O'Brien, J.S. (1974) *Analyt. Biochem.* **60**, 424-430.

21. DiMatteo, G., Orfeo, M.A. and Romeo, G. (1976) *Biochim. Biophys. Acta* **429**, 527-537.

22. Turner, B.M. (1979) *Biochim. Biophys. Acta* **578**, 325-336.

23. Robinson, D., and Thorpe, R. (1974) *FEBS Lett.* **45**, 191-193.

24. Alhadeff, J.A., Miller, A.L., Wenaas, H., Vedvick, T. and O'Brien, J.S. (1975) *J. Biol. Chem.* **250**, 7106-7113.

25. Beir, E.M., Klyashchitskii, B.A., and Vinderschain, G.Y. (1979) *Biochemistry (USSR)* **44**, 1535-1539.

26. Miyagawa, T., and Doi, F. (1979) *J. Invest. Derm.* **73**, 554-557.

27. Avila, J.L., and Convit, J. (1974) *Biochim. Biophys. Acta* **358**, 308-318.

28. Kolibaba, L.G., Rosenfield, E.L., and Wiederschain, G.Y. (1975) *Vopr. Med. Khim.* **21**, 637-644.

29. Troost, J., Van der Heijden, M.C.M. and Staal, G.E.J. (1976) *Clin. Chim. Acta* **73**, 321-327.

30. Turner, B.M., Beratis, N.G., Turner, V.G. and Hirschhorn, K.(1974) *Clin. Chim. Acta* **57**, 29-35.

31. Alhadeff, J.A. and Janowsky, A.J. (1978) *Clin. Chim. Acta* **82**, 133-140.

32. DiCioccio, R.A., Barlow, J.J., and Matta, K.L. (1982) *J. Bio.Chem.* **257**, 714-718.

33. Alhadeff, J.A. and Andrews-Smith, G.L. (1979) *Clin. Genet.* **16**, 157-163.

34. Bosmann, H.B. and Hemsworth, B.A. (1971) *Biochim. Biophys. Acta* **242**, 152-171.

35. Opheim, D.J. and Touster, O. (1977) *J. Biol. Chem.* **252**, 739-743.

36. Carlsen, R.B. and Pierce, J.G. (1972) *J. Biol. Chem.* **247**, 23-32.

37. Wright, K., Northcote, D.H. and Davey, R.M. (1976) *Carbohyd.Res.* **47**, 141-150.

38. Leray, G., Shacoori, V., Prodhomme, C., Jouanolle, A.M., and Blayau, M. (1988) *Arch. Andrology* **21**, 23-28.

39. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1987) *Biochem. J.* **245**, 589-593.

40. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1985) *Biochem. J.* **230**, 75-82.

41. Self, S.J., Winchester, B.G. and Archer, J.R. (1978) *Genet.Res.* **32**, 181-193.

42. Laury-Kleintop, L.D., Alhadeff, J.A., and Damjanov, I. (1985) *Dev. Biol.* **111**, 520-524.

43. Johnson, W.C., and Hong, J.L. (1986) *Biochem. Genet.* **24**, 469-483.

44. Alam, T., and Balasubramanian, A.S. (1978) *Biochim. Biophys. Acta* **524**, 373-384.

45. Alam, T., and Balasubramanian, A.S. (1979) *Biochim. Biophys. Acta* **566**, 327-334.

46. Grove, D.S., and Serif, G.S. (1981) *Biochim. Biophys. Acta* **662**, 246-255.

47. Wiederschain, G.Y. and Rosenfeld, E.L. (1971) *Biochim. Biophys. Res. Commun.* **44**, 1008-1014.

48. Wiederschain, G.Y., and Beyer, E.M. (1977) *Archs. Biochem. Biophys.* **182**, 335-342.

49. Jauhiainen, A. and Vanha-Perttula, T. (1986) *Biochim. Biophys.Acta* **880**, 91-95.

50. Srivastava, P.N., Arbtan, K., Takei, G.H., Huang, T.T.F. and Yanagimachi, R. (1986) *Biochim. Biophys. Res. Comm.* **137**, 1061-1068.

51. Barker, C., Dell, A., Rogers, M., Alhadeff, J.A., and Winchester, B. (1988) *Biochem. J.* **254**, 861-868.

52. Johnson, S.W., and Alhadeff, J.A. (1990) *Comp. Biochem. Physiol.* **97B**, 713-717.

53. Watkins, P. and Alhadeff, J.A. (1981) *Comp. Biochem. Physiol.* **68B**, 509-516.

54. Villar, E., Calvo, P. and Cabezas, J.A. (1978) *Comp. Biochem. Physiol.* **60B**, 459-461.

55. Hopfer, R.L., Johnson, S.W., Masserini, M., Giuliani, A. and Alhadeff, J.A. (1990) *Biochem. J.* **266**, 491-496.

56. Alhadeff, J.A., Tennant, L., and O'Brien, J.S. (1975) *Dev. Biol.* **47**, 319-324.

57. Alhadeff, J.A., Miller, A.L., Wenger, D.A. and O'Brien, J.S. (1974) *Clin. Chim. Acta* **57**, 307-313.

58. Thrope, R., and Robinson, D. (1975) *FEBS Lett.* **54**, 89-92.

59. Alhadeff, J.A., Andrews-Smith, G.L. (1980) *Biochim. Biophys. Acta* **614**, 466-475.

60. Alhadeff, J.A., Cimino, G., and Janowsky, A.J. (1978) *Mol. Cell. Biochem.* **19**, 171-180.

61. Kress, B.C., Freeze, H.H., Herd, J.K., Alhadeff, J.A., and Miller, A.L. (1980) *J. Biol. Chem.* **225**, 955-961.

62. Jain, R.S., Binder, R.L., Levy-Benshimol, A., Buck, C.A., and Warren, L. (1977) *J. Chromatogr.* **139**, 283-290.

63. Andrews-Smith, G.L., and Alhadeff, J.A. (1982) *Biochem. Biophys. Acta* **715**, 90-96.

64. Alhadeff, J.A., and Freeze, H. (1977) *Mol. Cell. Biochem.* **18**, 33-37.

65. Alhadeff, J.A., and Andrews-Smith, G.L. (1978) *Biochem. Med.* **20**, 357-363.

66. Fukushima, H., deWet, J.R., and O'Brien, J.S. (1985) *Proc. Nat. Acad. Sci. USA* **82**, 1262-1265.

67. O'Brien, J.S., Willems, P.J., Fukushima, H., deWet, J.R., Darby, J.K., DiCioccio, R., Fowler, M.L., and Shows, T.B. (1987) *Enzyme* **38**, 45-53.

68. Occhiodoro, T., Beckmann, K.R., Morris, C.P., and Hopwood, J.J. (1989) *Biochem. Biophys. Res. Commun.* **164**, 439-445.

69. Fisher, K.J., and Aronson, N.N., Jr. (1989) *Biochem. J.* **264**, 695-701.

70. Kretz, K.A., Cripe, D., Carson, G.S., Fukushima, H., and O'Brien, J.S. (1992) *Genomics* **12**, 276-280.

71. Barnes, A.K., and Wynn, C.H. (1988) *Protein. Struct. Funct. Genet.* **4**, 182-189.

72. Muller-Taubenberger, A., Westphal, M., Noegel, A., and Gerisch, G. (1989) *FEBS Lett.* **246**, 185-192.

73. Willems, P.J., Darby, J.K., DiCioccio, R.A., Nakashima, P., Eng C., Ketz, K.A., Cavalli-SForza, L.L., Shooter, E.M., and O'Brien, J.S. (1988) *Am. J. Hum. Genet.* **43**, 756-763.

74. Kretz, K.A., Darby, J.K., Willems, P.J., and O'Brien, J.S. (1989) *J. Mol. Neuro. Sci.* **1**, 177-188.

75. Willems, P.J., Gatti, R., Darby, J.K., ROmeo, G., Durand, P., Dumon, J.E., and O'Brien, J.S. (1991) *J. Med. Genet.* **38**, 111-131.

76. Alhadeff, J.A., and Andrews-Smith, G.L. (1979) *Biochem. J.* **177**, 753-756.

77. Argade, S.P., Hopfer, R.L., Strang, A.M., van Halbeek, H., and Alhadeff, J.A. (1988) *Arch. Biochem. Biophys.* **266**, 227-247.

78. Johnson, S.W., Piesecki, S., Wang, R.F., Damjanov, I., Alhadeff, J.A. (1992) *Biochem. J.* **282**, 829-834.

79. Thorpe, R., and Robinson, D. (1978) *Clin. Chim. Acta* **86**, 21-30.

80. Turner, B.M. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, abstract 2895.

CHAPTER TWO

RODENT TISSUE α-L-FUCOSIDASES: ANALYSIS OF BRAIN AND SPLEEN ISOFORMS

2.1. INTRODUCTION

Mouse liver α-L-fucosidase is different from other mammalian fucosidases studied to date because of the presence of isoelectric forms (isoforms) with pI values above 7.0 comprising approximately 25% of the total activity [1,2]. These isoforms are found in addition to the usual acidic and neutral forms described in tissues of other mammals [3]. The unusual properties of mouse α-L-fucosidase have prompted study of α-L-fucosidase derived from the livers of hamster, guinea-pig, and rat to determine whether the properties of α-L-fucosidase are similar in other members of the Order Rodentia [4] since differences in these properties reflect alterations in amino acid sequence and/or post-translational modifications [5]. The results indicated that α-L-fucosidase from hamster liver, but not from rat and guinea-pig livers, is similar to the mouse enzyme with respect to its isoform profile. Hamster liver enzyme contains three isoforms with pI values of 8.2, 9.0 and 9.8 in addition to other forms[4].

This chapter presents the results of a series of isoelectric focusing studies which provide some information on the relatedness of isoelectric forms from other tissues (spleen and brain) from four rodents (mouse, guinea-pig, rat, and hamster). These tissues have been examined to determine if they contain the unusual fucosidase isoforms above pI 7.0.

2.2. MATERIALS AND METHODS

2.2.1. GENERAL

All procedures were carried out at 2-4°C unless otherwise specified. Spleens and brains from mouse (*Mus musculus*, Swiss Webster strain), hamster (*Mesocricetus auratus*, Golden Syrian strain), rat (*Rattus norvegicus*) and guinea-pig (*Cavia porcellus*, Hartley strain) were purchased from Rockland, Gilbertsville, PA.

2.2.2. PREPARATION OF SUPERNATANT FLUIDS

Tissues (0.5-1.2 g) were homogenized 1:5 (w/v) with 10 mM, pH 5.5, NaH_2PO_4 containing 0.02% (w/v) NaN_3 (phosphate buffer) in a Potter-Elvehjem tissue grinder. The homogenates were centrifuged at 27,000 x g for 40 min and the resulting supernatant fluids were assayed for α-L-fucosidase activity using the synthetic substrate 4-methylumbelliferyl-α-L-fucopyranoside (4-MU-fucoside, Sigma Chemical Company, St Louis, MO) as described previously [4]. A unit of activity is defined as the amount of enzyme required to hydrolyze 1 nmol of substrate/min at 37°C.

2.2.3. ISOELECTRIC FOCUSING

Isoelectric focusing was performed at 2-4°C using a 40 ml column essentially as described previously [4] with 2% (v/v) ampholytes (pH range 5-7; Pharmacia LKB Biotechnology, Bromma, Sweden) and a 0-67% (w/v) sucrose gradient. Electrofocusing

was conducted at 600 V (1.5-2.0 mA) for 12-18 hr after which 0.4 ml fractions were collected. The pH value of each fraction was determined at 2-4°C using a Beckman Φ 21 digital display pH meter, and 50 μl aliquots of each fraction were assayed for 30 min at 37°C for α-L-fucosidase activity. The results were plotted as amount of activity vs pI and the relative percentages of α-L-fucosidase activity associated with the isoforms above and below pI 7.0 were determined by cutting out the respective portions of the profiles and weighing the peaks on a Mettler analytical balance.

2.3. RESULTS

Figure 2.1 (panel A) shows the results of isoelectric focusing of α-L-fucosidase from the supernatant fluid of Swiss Webster mouse spleen. Approximately 70% of the α-L-fucosidase activity is found in three to four isoforms below pI 7.0 while the remainder of the activity is contained in two to three isoforms with approximate pI values of 7.8, 8.4, and 9.2. The results of isoelectric focusing of α-L-fucosidase from guinea-pig, rat and hamster spleen supernatant fluids are given in panels B, C, and D, respectively, of Figure 2.1. The rat and hamster spleen profiles indicate that multiple isoforms exist for both enzymes but only hamster fucosidase contains significant activity (approx. 46% of total activity) above pI 7.0. Guinea-pig spleen α-L-fucosidase exhibits a profile showing a broad isoform centered around pI 5.3 with no activity above pI 6.0. The relative distributions of α-L-fucosidase activity above and below pI 7.0 for mouse, guinea-pig, rat and hamster spleen are summarized in Table 2.1.

Isoelectric focusing was also performed on α-L-fucosidase from mouse, guinea-pig, rat and hamster brain supernatant fluids (panels A, B, C, and D, respectively, in Figure 2.2). The profiles indicate that only mouse and hamster brain fucosidases contain significant activity above pI 7.0. The mouse and hamster isoforms above pI 7.0 comprise about 72% and 30%, respectively, of the total activity. The relative distributions of α-L-fucosidase activity above and below pI

7.0 for mouse, guinea-pig, rat and hamster brain are also summarized in Table 2.1. These results indicate that only hamster and mouse α-L-fucosidases contain isoforms above pI 7.0 in spleen and brain tissues.

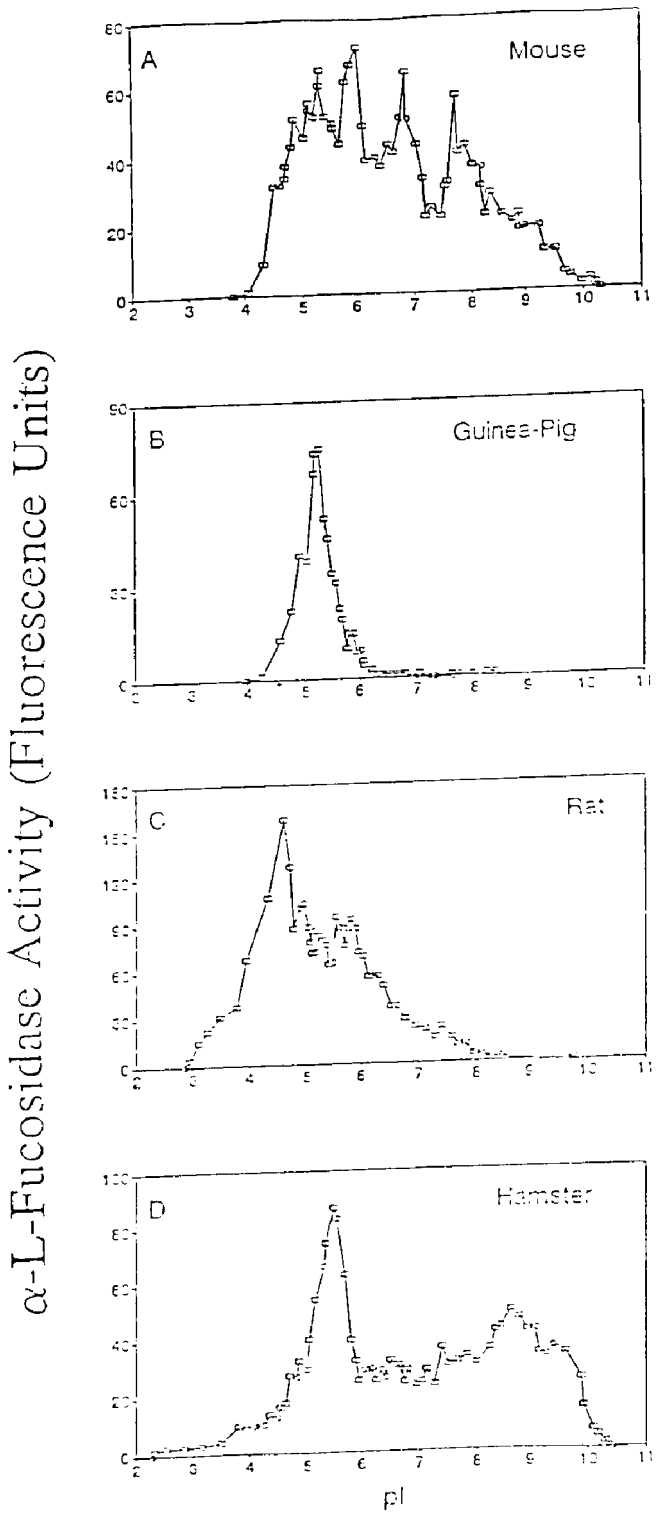


Figure 2.1: Isoelectric focusing profiles of spleen α-L-fucosidase from mouse (A), guinea-pig (B), rat (C), and hamster (D) supernatant fluids

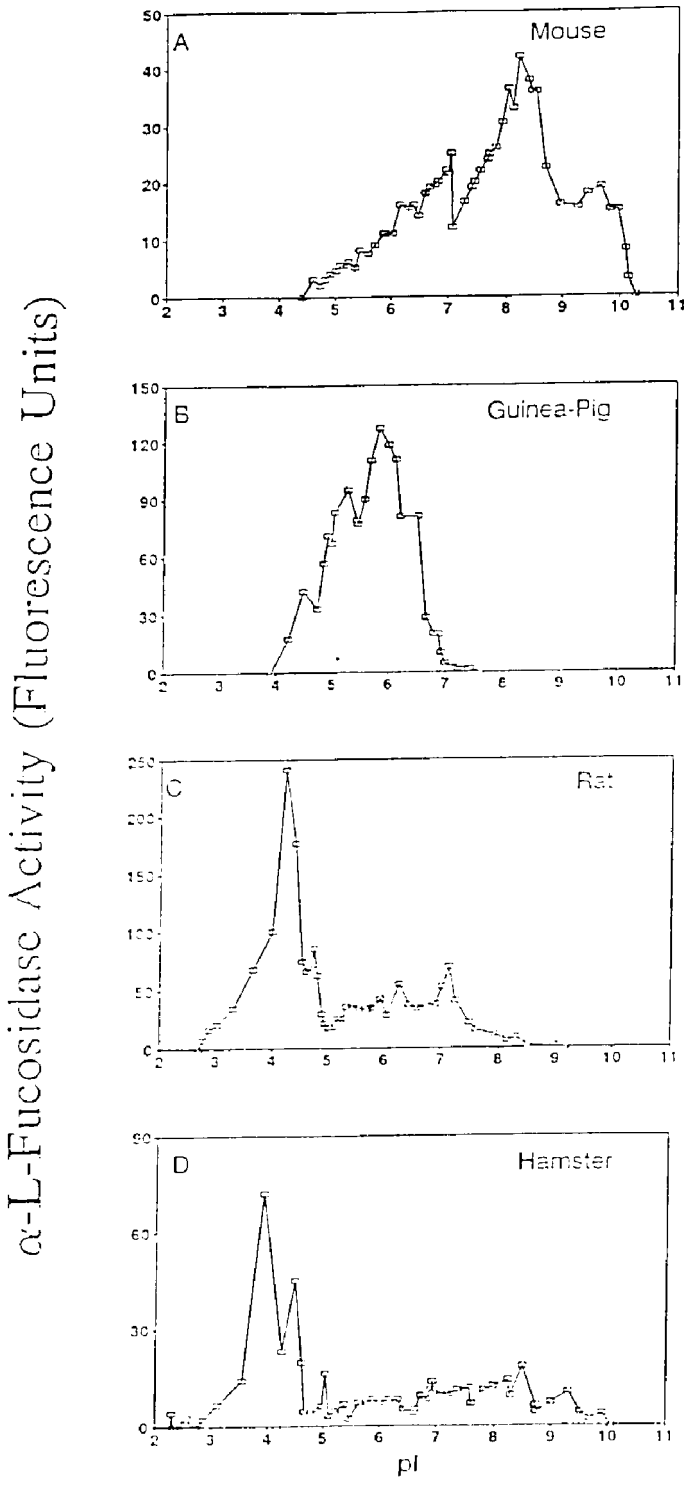


Figure 2.2: Isoelectric focusing profiles of brain α-L-fucosidase from mouse (A), guinea-pig (B), rat (C), and hamster (D) supernatant fluids.

Table 2.1: RELATIVE DISTRUBUTION OF RODENT SPLEEN AND BRAIN α-L-FUCOSIDASE ACTIVITY IN THE ISOELECTRIC FOCUSING PROFILES

TISSUE	SPECIES	STRAIN	RELATIVE DISTRIBUTION BELOW pI 7.0	% OF RECOVERED ACTIVITY ABOVE pI 7.0
spleen	mouse	Swiss Webster	70	30
spleen	guinea-pig	Hartley	100	0
spleen	rat		95	5
spleen	hamster	Golden Syrian	54	46
brain	mouse	Swiss Webster	28	72
brain	guinea-pig	Hartley	100	0
brain	rat		91	9
brain	hamster	Golden Syrian	70	30

2.4. DISCUSSION

A number of investigations have provided evidence for an unusual α-L-fucosidase in mouse tissues. A spermatozoal variant of fucosidase (AFuc-2) in inbred mice has been described with a pH optimum of 5.4 but significant activity at the low pH of 3.5 [6]. Variant α-L-fucosidases have been found in numerous tissues from inbred mouse strains that differ in their thermostability and relative activity at low pH (2.8) [7]. These latter authors concluded that two fucosidase variants exist in inbred mouse strains, and that these variants are probably genetically determined. Previous investigations in our laboratory [1,2] have indicated that mouse liver α-L-fucosidase has an isoform composition that differs from most mammalian α-L-fucosidases. Further studies [4] on three other rodents (guinea-pig, rat, hamster) liver α-L-fucosidases indicated that only the hamster liver enzyme was similar to the mouse enzyme with regard to the presence of significant amounts of isoforms with pI values above 7.0.

In the present investigation, the isoform profiles of α-L-fucosidase from rodent (mouse, guinea-pig, rat, hamster) spleen and brain tissues have been studied to determine whether the unusual features of the mouse and hamster liver enzymes are found in these other tissues. The current results suggest that fucosidase isoforms above pI 7.0 are present only in mouse and hamster spleen and brain. It thus appears from the present and past studies [1,2,4,8] that, of the rodents

studied, only mouse and hamster contain the atypical α-L-fucosidase with significant amounts of isoforms above pI 7.0 (summarized in Table 2.2). Earlier experiments in our lab [3] have indicated that rat liver α-L-fucosidase is the most similar of the rodent fucosidases to the human enzyme with respect to its isoform profile, K_m value, pH activity curve and binding to the fucosamine resin. The present investigation provides further evidence for this similarity: rat spleen and brain α-L-fucosidases appear to be the most similar of the rodent fucosidases to the human enzyme with respect to their isoform profiles. This is a reasonable finding since the amino acid sequence of rat liver α-L-fucosidase contains 82% sequence identity with human liver α-L-fucosidase [9]. However, it is important to note that studies on unpurified enzymes should be interpreted with caution since certain properties in crude tissue and fluid preparations may not reflect the enzyme's true properties (e.g. due to the presence of inhibitors, lack of cofactors, protein-protein interactions, etc.). At present, the biological significance of the atypical rodent fucosidase found in mouse and hamster (and not in rat and guinea-pig) is not understood. This atypical fucosidase, which is characterized by the presence of isoforms with significant activity above pI 7.0, may represent a variant form of the enzyme with an as yet unknown specialized function.

Table 2.2: SUMMARY OF PERCENT OF α-L-FUCOSIDASE IN RODENT TISSUE ISOFORM PROFILES ASSOCIATED WITH ACTIVITY ABOVE pI 7.0

RODENT	LIVER*	SPLEEN	BRAIN
Mouse	20-30%	30%	72%
Hamster	50%	46%	30%
Rat	7%	5%	9%
Guinea pig	0%	0%	0%

* The liver data are from a previous study [4].

2.5. REFERENCES

1. Laury-Kleintop, L.D., Damjanov, I. and Alhadeff, J.A. (1985) *Biochem. J.* **230**, 75-82.

2. Laury-Kleintop, L.D., Damjanov, I. and Alhadeff, J.A. (1987) *Biochem. J.* **245**, 589-593.

3. Johnson, S.W., and Alhadeff, J.A. (1991). *Comp. Biochem. Physiol.* **99B**, 479-488.

4. Johnson, S.W., and Alhadeff, J.A. (1990) *Comp. Biochem. Physiol.* **97B**, 713-717.

5. Paigen, K. (1979) *Ann. Rev. Genet.* **13**, 417-466.

6. Self, S.J., Winchester, B.G., and Archer, J.R. (1978) *Genet. Res.* **32**, 183-193.

7. Johnson, W.C. and Hong, J.L. (1986) *Biochem. Genet.* **24**, 469-483.

8. Shoarinejad, F., Johnson, S.W., and Alhadeff, J.A. (1993) *Comp. Biochem. Physiol.* **105B**, 129-137.

9. Fisher, K.J., and Aronson, N.N., Jr. (1989) *Biochem J.* **264**, 695-701.

CHAPTER THREE

PURIFICATION AND SUBUNIT ANALYSIS OF MOUSE LIVER α-L-FUCOSIDASE

3.1. INTRODUCTION

Previous studies have indicated that a novel α-L-fucosidase is present in mouse tissues and cells [1-4]. The mouse enzyme contains a significant percentage (20-25%) of its total activity in unique basic isoforms in addition to the usual acidic and neutral isoforms found in other mammalian species [5]. Mouse α-L-fucosidase has a subunit composition (63 and 57 kDa) different from that of human α-L-fucosidase (56 and 51 kDa) [6]. The similar M_rs of the mouse 57 kDa subunit and the human 56 kDa subunit raise the possibility that mouse and human fucosidases may share a common subunit while each has a unique subunit.

This chapter presents results of a series of experiments which have been conducted to determine the structural relatedness of the separated subunits of purified mouse liver α-L-fucosidase. In addition, these results have been compared to the results of similar studies on human liver α-L-fucosidase [7].

3.2. MATERIALS AND METHODS

3.2.1. GENERAL

All procedures were carried out at 2-4° C unless otherwise stated. The method of Lowry et al. [8] was used for protein determination, with human serum albumin (Fraction V. Sigma Chemical Co., St. Louis, MO) as the standard. Mouse livers were purchased from Rockland, Inc. (Gilbertsville, PA) and stored at -20° C until used.

3.2.2. PURIFICATION OF HUMAN LIVER α-L-FUCOSIDASE

α-L-Fucosidase was purified from human liver to apparent homogenity by affinity chromatography using agarose-ε-aminohexanoyl fucosamine resin (Miles Scientific, Naperville, IL) as described elsewhere [9].

3.2.3. PREPARATION OF SUPERNATANT FLUID

Mouse liver (*Mus musculus,* Swiss Webster strain, 500 g) (Rockland, Gilbertsville, PA) was homogenized 1:5 (w/v) in 10 mM NaH_2PO_4 buffer, pH 5.5, containing 0.02% NaN_3 (phosphate buffer) in a Waring blender. Following centrifugation at 27,000 x g for 40 min, the resulting supernatant was filtered through glass wool/cheese cloth and dialyzed for 3 days against 75 liters of 10 mM NaH_2PO_4 buffer (phosphate buffer). The dialyzate was centrifuged at 27,000 x g for 40 min and the resulting supernatant was filtered through a glass wool/cheese

cloth filter. The homogenate and supernatant fluids were assayed for α-L-fucosidase activity under linear conditions using the 4-MU-fucoside substrate.

3.2.4. PREPARATION OF IMMUNOAFFINITY RESIN (GOAT-ANTI-HUMAN-α-L-FUCOSIDASE IgG-SEPHAROSE 4B)

This resin was prepared essentially as described elsewhere [6]. The frozen goat-anti-human liver α-L-fucosidase-IgG (1 ml) was diluted 1:30 with 0.1 M $NaHCO_3$, pH 8.0 and mixed with 2 g of CNBr-activated Sepharose 4B (Sigma Chemical Co. St. Louis, MO, U.S.A.). The slurry was stirred at 2-4° C for 72 hrs. Twenty five ml of 1.0 M ethanolamine, pH 8.0 was added and the suspension was stirred overnight at 2-4° C. The resin was washed alternatively with 40 ml of 0.1 M $NaHCO_3$, pH 8.0 containing 0.5 M NaCl and 0.1 M NaOAc, pH 4.0 containing 0.5 M NaCl for a total of five washes in a Buchner funnel. The resin was then washed and resuspended with 25 ml of 10 mM NaH_2PO_4/0.15 M NaCl (PBS), pH 5.5 containing 0.02% NaN_3 and stored at 2-4° C.

3.2.5. PURIFICATION OF MOUSE LIVER α-L-FUCOSIDASE

Mouse liver supernatant was routinely applied to a column containing 4 ml of immunoaffinity resin at a flow rate of 15-20 ml/hr until the α-L-fucosidase activity in the run-through was equal to that of the supernatant fluid. The column was washed with several liters of 10 mM NaH_2PO_4/0.15 M NaCl, pH

5.5, containing 0.02% NaN_3 (PBS) until the absorbance readings at 280 nm were less than 0.005. The fucosidase was eluted with 25 mM fucose (Sigma) in 70% (v/v) ethylene glycol (instead of 50% ethylene glycol used previously, [6]) in 10 mM PBS, pH 8.2, containing 0.02% NaN_3. Fractions (4.5 ml) were collected in tubes containing 1.5 ml of 0.1 M sodium citrate/citric acid, pH 5.0, containing 0.02% NaN_3, and those fractions containing α-L-fucosidase activity were pooled and concentrated by ultrafiltration using Amicon YM-10 membranes and Centricon 10 microconcentrators (Amicon Corp., Lexington, MA). The purified α-L-fucosidase was assayed with the fluorescent substrate 4-methylumbelliferyl-α-L-fucopyranoside (Sigma, St. Louis, MO) in the presence of 5 mg/ml human serum albumin (HSA) to stabilize the enzyme.

3.2.6. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Slab SDS-PAGE was used, according to the method of Laemmli [10], to assess purity and characterize the subunits of purified human and mouse liver α-L-fucosidase. Enzyme samples (2-26 μg) were incubated for 5-8 min at 100° C in a sample buffer containing 2.0% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 62.5 mM, pH 6.8, Tris-HCl, 10% (v/v) glycerol and 0.0012% (w/v) Bromophenol Blue. Samples were electrophoresed on a 0.1% SDS gel (composed of a 4.0% stacking gel and a 10% separating gel) at 150 V for 1 hr at 4.0° C in

a pH 8.6 running buffer containing 25 mM Tris, 0.2 M glycine and 0.1% SDS. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA) prepared in methanol/acetic acid/ H_2O (2:3:35, v/v/v), and gels were destained with methanol/acetic acid/H_2O (2:3:35, v/v/v). The Laemmli method was also used to separate the subunits of mouse α-L-fucosidase for lectin blotting (see below).

The method of Plaxton and Moorhead [11] was used to separate mouse liver α-L-fucosidase from minor protein contaminants for immunoblotting analysis. Mouse liver α-L-fucosidase samples (13 μg) were incubated for 5 min at 100° C in 70 mM Tris-HCl (pH 6.7) buffer containing 8 M urea, 3.0% (w/v) SDS, 100 mM dithiothreitol and 0.005% (w/v) Bromophenol Blue. Samples were electrophoresed in a discontinuous system composed of a 4% stacking gel and a 10% separating gel [75:2 (w/w) acrylamide:bisacrylamide] at 150 V at room temperature for 1 hr in an upper buffer containing 0.1% (w/v) SDS, 100 mM Tris-150 mM glycine (no pH adjustment) and a lower buffer made from a 1:1 (v/v) dilution of the upper buffer. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) prepared in methanol/acetic acid/H_2O (2:3:35, v/v/v) for 15 min and destained in methanol/H_2O (1:1, v/v). Bands corresponding to the mouse liver α-L-fucosidase subunits were excised with a razor blade from the 10% separating gels and each gel slice was cut in half so as to approximate the width

of the well for a subsequent 12% separating gel [75:2 (w/w) acrylamide:bisacrylamide]. Each gel slice was then gently transferred to a sample well of a 1.0-mm thick slab mini-gel prepared according to the method of Plaxton with a Teflon spatula until the gel slice rested against the bottom of the sample well. Gels were electrophoresed at 150 V for 1.5 hr.

3.2.7. WESTERN BLOTTING OF THE SUBUNITS OF α-L-FUCOSIDASE

Following SDS-PAGE, the gels were allowed to equilibrate for 30 min in 25 mM Tris and 192 mM glycine in 20% (v/v) methanol. The protein was transferred to 0.2 μm nitrocellulose (Schleicher and Schuell, Keene, NH) at 100 V for 1.5 hr using a Bio-Rad Mini-Trans Blot electrophoresis cell according to the method described in the manual accompanying the unit. A portion of the nitrocellulose was stained for 1 min with 0.1% (w/v) Amido Black 10B (Bio-Rad) in 2-propanol/acetic acid/H_2O (3:1:6, v/v/v). The remainder of the nitrocellulose was incubated in the appropriate Tris buffer for immunodetection (see below).

3.2.8. IMMUNODETECTION

3.2.8.1. PAb's and MAb's

The nitrocellulose was incubated in 10 mM, pH 7.5, Tris-HCl containing 0.15 M NaCl and 0.1% Tween 20 (TBST) for 12 hrs. The nitrocellulose was incubated with either a 10^3 or a 10^4 dilution of goat-anti-human liver α-L-fucosidase

polyclonal antibodies (PAb's) [12], a 10^3 or 10^4 dilution of monoclonal antibody (MAb) #116, a 10^2 dilution of MAb #100, a 10^2 dilution of MAb #72, a 10^2 dilution of MAb #40, in TBST for 30-60 min (polyclonal, MAb #116) or 10-17 hrs (MAb #72, MAb #100, MAb #40) [7]. The samples were subsequently washed three times in TBST (10 min/wash) followed by a 30-60 min incubation with a $5x10^3$ dilution of secondary antibody, (either rabbit-anti-goat IgG for PAb or goat-anti-rat IgG/IgM for MAb). Both secondary antibodies were available coupled to alkaline phosphatase (Jackson Immunoresearch, West Grove, PA). Following three more 10 min washes in TBST, the nitrocellulose samples were placed into color developing buffer [37.5 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml dimethylformamide, DMF) and 50 μl Nitro Blue tetrazolium, NBT (75 mg/ml 70% DMF) in 10 ml of 0.1 M, pH 9.5, Tris-HCl containing 0.1 M NaCl and 5 mM $MgCl_2$] for 2-30 min. The color development reactions were stopped by immersing the nitrocellulose in milli-Q H_2O.

3.2.8.2. LECTINS

The nitrocellulose was incubated in 10 mM, pH 7.5, Tris-HCl containing 0.15 M NaCl (TBS) for 12 hrs. The nitrocellulose was then incubated for 1 hr in 0.5% (w/v) blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN) in TBS. After being washed twice with TBS and once with TBS containing 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM

$CaCl_2$, pH 7.5, the membrane was incubated for 1 hr at room temperature with lectin-digoxigenin conjugates (*Galanthus nivalis agglutinin*, GNA; *Sambucus nigra agglutinin*, SNA; Boehringer Mannheim Biochemicals, Indianapolis, IN) at a concentration of 0.1 μg lectin-digoxigenin conjugate/ml of 50 mM, pH 7.5 TBS containing 0.05% (w/v) NaN_3, and 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$. The nitrocellulose was subsequently washed three times with TBS followed by a 1 hr incubation with a 10^3 dilution of the Fab fragment polyclonal sheep anti-digoxigenin coupled to alkaline phosphatase. Following three more washes in TBS (10 min/wash) the nitrocellulose was placed in color developing buffer as described above.

3.3. RESULTS

Human liver α-L-fucosidase was purified 7500-fold by affinity chromatography using the fucosamine resin [9] to a final specific activity of 14,500 U/mg protein with an 80% yield. Mouse liver α-L-fucosidase was purified 18,700 fold by immunoaffinity chromatography to a final specific activity of 7500 U/mg protein with a 66% yield. The improved yield for the immunoaffinity procedure over that previously obtained (15%) [6] resulted from increasing the concentration of ethylene glycol in the elution buffer from 50 to 70% (v/v).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to nitrocellulose and detection of protein with Amido Black, indicated that mouse α-L-fucosidase contained two major bands near 57 and 62 kDa and a few minor bands (Fig. 3.1, lane 3). Human fucosidase, run as a control, contained bands at 51 and 56 kDa (Fig. 3.1, lane 2). The polyclonal antibody (PAb) appears to recognize both human bands (Fig. 3.1, lane 4), and at least the 57 kDa mouse band (Fig. 3.1, lane 5).

Western blotting of mouse liver α-L-fucosidase (Fig. 3.2) indicated that monoclonal antibodies (MAbs) #40 (lane 3) and #72 (lane 4) recognized at least the 62 kDa protein band whereas MAb #116 (lane 5) appears to recognize both protein bands. The narrower banding pattern seen with MAbs #40 and #72 (when compared to MAb 116) may be due to decreased recognition or non-recognition of the 57 kDa mouse band. The vertical line

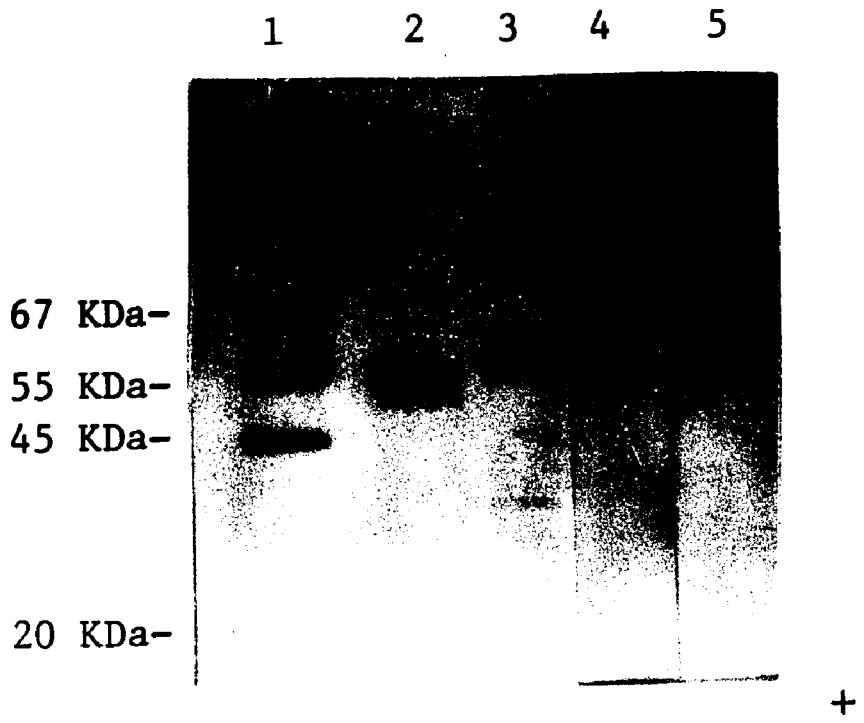


Figure 3.1: Western blots of purified human liver α-L-fucosidase and mouse liver α-L-fucosidase with polyclonal antibodies. See Materials and Methods for details.

Lane 1. Standard protein M_r markers: 2 μg bovine serum albumin (67 kDa), 4 μg gamma globulin (55 and 20 kDa), and 2 μg ovalbumin (45 kDa) stained with 0.1% (w/v) Amido Black.

Lane 2. 2 μg Human liver α-L-fucosidase stained with 0.1% (w/v) Amido Black.

Lane 3. 4 μg Mouse liver α-L-fucosidase stained with 0.1% (w/v) Amido Black.

Lane 4. 0.5 μg Human liver α-L-fucosidase detected with a 10^3 dilution of PAb.

Lane 5. 4 μg Mouse liver α-L-fucosidase detected with a 10^3 dilution of PAb.

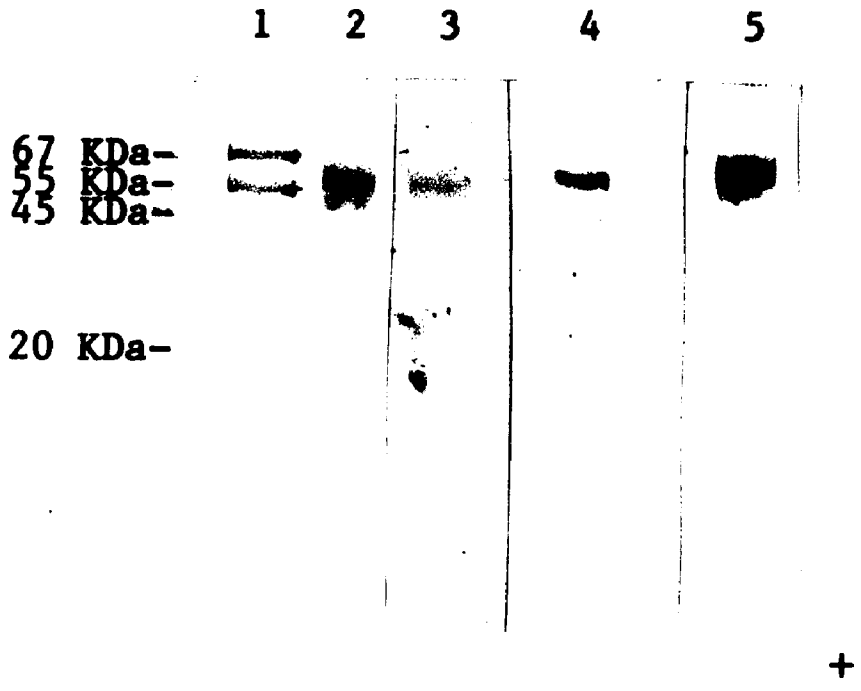


Figure 3.2: Western Blot of purified mouse liver α-L-fucosidase (5 μg/lane) with monoclonal antibodies. See Materials and Methods for details.

Lane 1. Standard protein M_r markers: 2.7 μg bovine serum albumin (67 kDa), 5.2 μg gamma globulin (55 and 20 kDa), and 2.7 μg ovalbumin (45 kDa) stained with 0.1% (w/v) Amido Black.

Lane 2. Mouse liver α-L-fucosidase stained with 0.1% (w/v) Amido Black.

Lane 3. Mouse liver α-L-fucosidase detected with 10^2 dilution of MAb#40.

Lane 4. Mouse liver α-L-fucosidase detected with 10^2 dilution of MAb#72.

Lane 5. Mouse liver α-L-fucosidase detected with 10^4 dilution of MAb#116.

in lane 3 is an artifact caused by a stir bar during development of the blot.

Figure 3.3 shows a Western blot analysis of the subunits of mouse liver α-L-fucosidase using digoxigenin-labeled lectins. The subunits were separated by SDS-PAGE, sliced from the gel after staining briefly with coomassie and run separately on another SDS-PAGE. The samples were analyzed by digoxigenin-labeled lectins with specificity for sialic acid linked α2-6 to galactose (Gal) (*Sambucus nigra agglutinin, SNA*) and for terminal mannose (Man) linked α1-2, α1-3 or α1-6 to Man (*Galanthus nivalis agglutinin, GNA*). The results indicated that both the high- and low-M_r subunits were recognized by SNA (lanes 1 and 2) and by GNA (lanes 3 and 4). The faint bands in lanes 1 and 2 may be the result of underdevelopment during the color reaction.

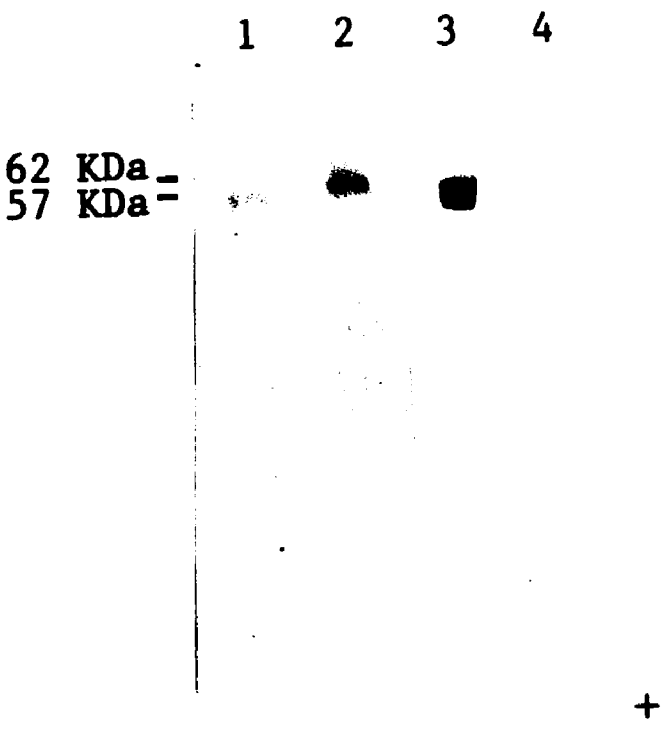


Figure 3.3: Lectin blot of separated mouse liver α-L-fucosidase subunits (3 μg/lane). See Materials and Methods for details.

Lane 1. 57 kDa mouse subunit detected with *SNA*.
Lane 2. 62 kDa mouse subunit detected with *SNA*.
Lane 3. 57 kDa mouse subunit detected with *GNA*.
Lane 4. 62 kDa mouse subunit detected with *GNA*.

3.4. DISCUSSION

Mouse liver α-L-fucosidase is different from other characterized mammalian fucosidases because of the presence of basic isoforms [1-4], which appear to be developmentally regulated and related to the usual neutral and acidic isoforms by the amount of sialytion [1,3]. The mouse enzyme is also unusual in that it does not bind well to the fucosamine affinity resin which has been widely used to purify mammalian fucosidases [9,13-17].

In the present investigation Western blotting analysis has been employed to study the structural relatedness of the separated subunits of purified mouse liver α-L-fucosidase and these results have been compared to recent results from human liver α-L-fucosidase [7]. The subunit composition of mouse fucosidase was similar to previous results [6] and contained two dissimilar subunits of 57 and 62 kDa. Western blotting analysis indicated that both of the mouse subunits were immunoreactive with PAb's and the three MAb's raised against human α-L-fucosidase. This suggests that the 62 kDa mouse subunit is not unique and shares some sequence identity with at least one of the human subunits. The presence of minor non-immunoreactive protein bands in the mouse fucosidase preparation may be due to very small amounts of non-specifically bound contaminating proteins which are eluted from the immunoaffinity column by the ethylene glycol. The mouse fucosidase used for the blotting studies was separated

from the minor contaminants by preparative SDS-PAGE.

Western analysis of fucosidase using lectins indicated that both mouse subunits are *GNA*-positive and *SNA*-positive. This evidence indicates that both 62 and 57 kDa subunits are glycosylated and suggests that both subunits contain the sialylated N-acetyllactosamine-type oligosaccharides along with oligomannoside-type chains. However, this is different from human fucosidase where the low-M_r human subunit was positive with *GNA* whereas the high-M_r human subunit was positive with both *GNA* and *SNA* [7].

In summary, purified mouse liver α-L-fucosidase exhibits differences from human liver α-L-fucosidase in its subunit composition, its interaction with MAbs (prepared against the human enzyme), and its interaction with the sialic acid binding lectin, *SNA*. However, these structural and antigenic differences have little effect on the kinetic and substrate specificity properties of mouse fucosidase, which are very similar to the properties of human fucosidase [18].

3.5. REFERENCES

1. Laury-Kleintop, L.D., Alhadeff, J.A., and Danjanov, I. (1985) *Devl. Biol.* **111**, 520-524.

2. Laury-Kleintop, L.D., Alhadeff, J.A., and Damjanov, I. (1985) *Br. J. Cancer* **5**, 949-951.

3. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1985) *Biochem. J.* **230**, 75-82.

4. Johnson, S.W., and Alhadeff, J.A. (1990) *Comp. Biochem. Physiol.* **97B**, 713-717.

5. Alhadeff, J.A., and O'Brien, J.S. (1977) *In Practical Enzymology of the Sphingolipidoses* (Edited by Glew R.H., and Peters, S.P.), pp. 247-281, Alpn R. Liss, New York.

6. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1987) *Biochem. J.* **245**, 589-593.

7. Johnson, S.W., Piesecki, S., Wang, R.F., Damjanov, I., and Alhadeff, J.A. (1992) *Biochem. J.* **282**, 829-834.

8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275.

9. Alhadeff, J.A., Miller, A.L., Wenass, H., Vedvick, T., and O'Brien, J.S. (1975) *J. Biol. Chem.* **250**, 7106-7113.

10. Laemmli, U.K. (1970) *Nature* **227**, 680-685.

11. Plaxton, W.C., and Moorhead, G.B.G. (1989) *Analyt. Biochem.* **178**, 391-393.

12. Andrews-Smith, G.L., and Alhadeff, J.A. (1982) *Biochim. Biophys. Acta* **715**, 90-96.

13. DiMatteo, G., Orfeo, M.A., and Romeo, G. (1976) *Biochim. Biophys. Acta* **429**, 527-537.

14. Wright, K., Northcote, D.H., and Davey, R.M. (1976) from *Zeamays L. Carbohydr. Res.* **47**, 141-150.

15. Opheim, D.J., and Touster, O. (1977) *J. Biol. Chem.* **252**, 739-743.

16. Chien, S.F., and Dawson, G. (1980) *Biochim. Biophys. Acta* **614**, 476-488.

17. Dicioccio, R.A., Barlow, J.J., and Matta, K.L. (1982) *J. Biol. Chem.* **257**, 714-718.

18. Shoarinejad, F., Johnson, S.W., and Alhadeff, J.A. (1993) *Comp. Biochem. Physiol.* **105B**, 129-137.

CHAPTER FOUR

PURIFICATION AND CHARACTERIZATION STUDIES ON HAMSTER LIVER α-L-FUCOSIDASE

4.1. INTRODUCTION

The discovery that mouse α-L-fucosidase has an isoform composition different from most mammalian α-L-fucosidases [1,2] has raised the question of whether this property is common to other members of the Order Rodentia. Further studies on three other rodent (rat, guinea-pig, hamster) liver α-L-fucosidases indicated that only the hamster liver enzyme is similar to the mouse enzyme with regard to the presence of significant amounts of isoforms with pI values above 7.0 [3].

In addition, as described in chapter 2, fucosidase isoforms above pI 7.0 are present only in mouse and hamster spleen and brain. The overall results indicate that, of the rodents studied, only mouse and hamster have the atypical α-L-fucosidase with significant amounts of isoforms above pI 7.0.

Since mouse liver α-L-fucosidase has already been purified and characterized, it would be of interest to further the analysis of atypical rodent fucosidase by characterizing the enzyme from hamster liver.

To characterize and to determine the structural relationship of hamster liver α-L-fucosidase to other mammalian fucosidases, specifically human and mouse, the hamster enzyme must first be purified.

α-L-Fucosidase has been purified by several research groups using conventional techniques of ammonium sulfate precipitation, gel filtration and ion exchange to isolate fucosidase from monkey brain, rat epididymis and rat cerebral

cortex [4-6]. However, the majority of the purification procedures used to isolate fucosidase involved the use of substrate-analogue affinity chromatography. The resin, agarose-ε-aminocaproyl-fucopyranosylamine, (Miles Scientific Naperville, IL) can reduce the purification to a one step procedure. Using fucosamine resin, the enzyme has been purified from human placenta [7,8], liver [9,10], brain [14], and spleen [12]; rat liver [13], porcine thyroid [11], and rat epididymis [15] by substrate-analogue affinity chromatography. This type of affinity chromatography, has resulted in large purification factors with relatively high yields [16].

Because of the success of using the fucosamine resin to purify mammalian fucosidases, an attempt was made to purify hamster liver fucosidase in this manner. This chapter describes the purification of hamster liver α-L-fucosidase. In addition, the results of characterization studies on purified enzyme, with regard to its isoform profile, pH optimum, subunit composition, and its antigenic relatedness to mouse and human liver α-L-fucosidase are presented.

4.2. MATERIALS AND METHODS

4.2.1. GENERAL

All procedures were carried out at 2-4° C unless otherwise specified. Hamster livers (*Mesocricetus auratus*, Golden Syrian strain) were purchased from Rockland, Gilbertsville, PA.

4.2.2. ISOELECTRIC FOCUSING

Isoelectric focusing was performed at 2-4° C using a 40-ml column essentially as described previously [3] with 2% (v/v) ampholytes, (pH range 5-7; Pharmacia LKB Biotechnology, Bromma, Sweden) and a 0-67% (w/v) sucrose gradient. Electrofocusing was conducted at 600 V (1.5-2.0 mA) for 12-18 hr after which 0.4 ml fractions were collected. The pH value of each fraction was determined at 2-4° C using a Beckman Φ 21 digital display pH meter, and 50 μl aliquots of each fraction were assayed for 30 min at 37° C for α-L-fucosidase activity. The results were plotted as amount of activity vs pI.

4.2.3. pH-ACTIVITY STUDIES

The pH activity curve was determined as described previously [3] by incubating 20 μl of purified enzyme with 30 μl of 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 5.8-8.0) or 0.1 M sodium citrate/citric acid (pH 3.0-6.2) and 100 μl 0.5 M 4-MU-fucoside in H_2O for 30 min at 37° C. Reactions were performed

in duplicate and the actual pH of the tissue blank was recorded.

4.2.4. PURIFICATION OF HAMSTER LIVER α-L-FUCOSIDASE

α-L-Fucosidase was purified from hamster liver by affinity chromatography using agarose-ε-aminocaproyl-fucosamine resin (Miles Scientific, Naperville, IL) essentially as described for human liver [9]. Hamster liver (*Mesocricetus auratus*, Golden Syrian strain, 100 g) (Rockland Inc., Gilbertsville, PA) was homogenized 1:5 (w/v) in a Waring blender with 10 mM NaH_2PO_4, pH 5.5, containing 0.02% (w/v) NaN_3. Following centrifugation at 27,500 x g for 40 min, the resulting supernatant fluid was filtered through glass wool/cheese cloth and dialyzed for 3 days against 25 l 10 mM NaH_2PO_4 buffer with a change of buffer each day. The dialysate was centrifuged at 27,500 x g for 40 min, filtered through glass wool/cheese cloth, and applied to the affinity column (2.0 x 4.0 cm) at a flow rate of 15-20 ml/hr. The amount of activity which bound was determined by assaying the column effluent using 4-MU-fucoside. The column was washed with 3 l of 10 mM NaH_2PO_4, pH 5.5, containing 0.15 M NaCl and 0.02% NaN_3 until the absorbance readings at 280 nm were less than 0.005. The fucosidase was eluted with 50 mM fucose (Sigma) in the pH 5.5, 10 mM phosphate buffer containing 0.15 M NaCl and 0.02% NaN_3. Fractions (4.5 ml) were collected and those fractions containing α-L-fucosidase activity were pooled and

concentrated by ultra-filtration using Amicon YM-10 membranes and Centricon 10 microconcentrators (Amicon Corp., Lexington, MA).

4.2.5. PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO HUMAN LIVER α-L-FUCOSIDASE

Monoclonal antibodies were prepared by Rong Fu Wang and Ivan Damjanov (Department of Pathology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA) by the standard hybridoma technique using spleen cells of immunized rats and the sp2/0 mouse myeloma cells for fusion according to the protocol of Fazekas de st. Groth and Scheideger [17], which was slightly modified for laboratory purposes [18]. Three rats were immunized four times at weekly intervals with 10-20 μg of purified human α-L-fucosidase. The rat that showed the strongest immunoreactivity with the α-L-fucosidase when dot-blotted to nitrocellulose sheets was used as a donor of spleen cells. Following the fusion, antibody secreting hybridomas were selected, and cloned and four hybridomas were expanded. Antibodies were collected as supernatant spent medium of cells grown *in vitro* or as the ascites fluid obtained in nude mice injected with 5 x 10^6 hybridomas. The monoclonal antibodies (MAb's) were isotyped using a Zymed MonoAb-ID ElA kit for rat monoclonals (Zymed, San Francisco, CA).

4.2.6. DOT IMMUNOBLOTTING

Purified human (1.5 μg) and hamster (1.5 μg) liver α-L-fucosidases were applied to 0.2 μm nitrocellulose sheets (Schleicher & Schuell, Keene, N.H.) using a Bio-Dot microfiltration apparatus (Bio-Rad). The samples were incubated in 10 mM, pH 7.4, Tris-HCl containing 0.15 M NaCl and 0.1% Tween 20 (Tris buffer) for 1 hr. The nitrocellulose was cut into strips and incubated with either a 10^3 dilution of goat-anti-human liver α-L-fucosidase polyclonal antibodies (PAb's) [19], a 10^3 dilution of monoclonal antibody (MAb) # 116, a 10^2 dilution of MAb # 100, a 10^2 dilution of MAb # 72, or a 10^2 dilution of MAb # 40 in Tris buffer for 12 hr. The samples were subsequently washed three times in Tris buffer (10 min/wash) followed by a 30 min incubation with a 5 x 10^3 dilution of secondary antibody, (rabbit-anti-goat IgG for PAb or goat-anti-rat IgG/IgM for MAb). Both secondary antibodies were coupled to alkaline phosphatase (Jackson Immunoresearch, West Grove, PA). Following three more 10 min washes in Tris buffer, the nitrocellulose samples were placed into developing buffer (3.3 mg Nitro Blue Tetrazolium and 1.7 mg 5-bromo-4-chloro-3-indolyl phosphate in 10 ml of 0.1 M, pH 9.5, Tris-HCl containing 0.1 M NaCl and 5 mM $MgCl_2$) for 5-30 min. The color development reactions were stopped by immersing the nitrocellulose in milli-Q H_2O.

4.2.7. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Slab SDS-PAGE was carried out according to the method of Laemmli [20] to assess purity and to characterize the subunits of purified hamster liver α-L-fucosidase. Enzyme samples (6-20 μg) were incubated for 5-8 min at 100° C in 62.5 mM Tris-HCl, pH 6.8, containing 2.0% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.0012% (w/v) Bromophenol Blue. Samples were electrophoresed on a 0.1% (w/v) SDS gel (composed of a 4% stacking gel and a 12% separating gel) at 100 V for 5-6 hr at 4° C in a pH 8.6 running buffer containing 25 mM Tris, 0.2 M glycine and 0.1% (w/v) SDS. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA) prepared in methanol/acetic acid/H_2O (2:3:35; v/v/v), and gels were destained with methanol/acetic acid/H_2O (2:3:35; v/v/v). Molecular weight standards included bovine serum albumin (67 kDa), IgG (55 and 20 kDa) and ovalbumin (45 kDa), and were purchased from Sigma.

4.2.8. WESTERN BLOT ANALYSIS OF THE SUBUNITS OF α-L-FUCOSIDASE

After SDS-PAGE, the gels were allowed to equilibrate for 30 min in 25 mM Tris and 192 mM glycine in 20% (v/v) methanol. The protein was transferred to 0.2 μm-pore-size nitrocellulose (Scheicher and Schuell, Keene, NH), at 100V for 1 hr by using a Bio-Rad Mini-Trans-Blot electrophoresis cell according to

the method described in the unit. The nitrocellulose was incubated in 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.1% Tween 20 (TBST) for 12 hr. The nitrocellulose was incubated with either a 10^3 or 10^4 dilution of PAb [19], or a 10^3 or 10^4 dilution of MAb # 116 [21], in TBST for 30-60 min. The samples were subsequently washed three times in the TBST (10 min/wash) followed by a 30-60 min incubation with a 5 x 10^3 dilution of rabbit-anti-goat IgG antibody for PAb, or goat anti rat IgG/IgM antibody for MAb. The secondary antibodies were coupled to alkaline phosphatase (Jackson Immunoresearch, West Grove, PA, and Boehringer-Mannheim Biochemicals). After three more 10-min washes in TBST, the nitrocellulose samples were placed into developing buffer (3.3 mg of Nitro-Blue Tetrazolium and 1.7 mg of 5-bromo-4-chloroindol-3-yl phosphate in 10 ml of 0.1 M Tris-HCl buffer, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$) for 2-3 min. The color development reactions were stopped by immersing the nitrocellulose in milli-Q H_2O.

4.3. RESULTS

Hamster liver α-L-fucosidase was purified approximately 57,000-fold with an 83% yield (relative to amount of activity in the supernatant fluid) to a final specific activity of 24,700 units/mg by an affinity chromatographic procedure [9] (Table 4.1). Approximately 80% of the α-L-fucosidase activity which was applied to the column was bound to the resin. Following elution with 50 mM fucose and further concentrating the fractions, approximately 2245 units of α-L-fucosidase (24% yield) were present with a specific activity of 24,664 units per mg protein.

Four hybridomas secreting antibodies against α-L-fucosidase enzyme were produced (using spleen cells), selected and cloned. The monoclonal antibodies (MAb's) produced by these hybridomas were isotyped, and two clones (#'s 40 and 100) were found to produce IgM whereas the other two clones (#'s 72 and 116) produced IgG 2a.

Dot immunoblotting of human and hamster α-L-fucosidase (Figure 4.1) with the polyclonal antibodies (PAb) and the four MAb's suggested that the MAb's might not be specifically recognizing hamster liver α-L-fucosidase since the secondary antibody (goat-anti-rat IgG/IgM) alone recognized hamster liver α-L-fucosidase (row 6, column Ha) but not human α-L-fucosidase (row 6, column Hu). The results suggested that the MAb's recognize human but not hamster α-L-fucosidase. Figure 4.2 depicts the results of isoelectric focusing of purified

TABLE 4.1: PURIFICATION OF HAMSTER LIVER α-L-FUCOSIDASE BY SUBSTRATE-ANALOGUE AFFINITY CHROMATOGRAPHY

Fraction	Activity (units)[a]	Protein (mg)	Specific activity units/mg protein	Purification factor	% yield
Hamster liver homogenate	9522	21,987	0.43	1.0	100
Supernatant	2693	13,380	0.20	0.47	28
Dialyzed, centrifuged supernatant	3105	8543	0.36	0.84	33
Purified enzyme	2245	0.090	24,944	58,010	24 (72)[b]

a. A unit of activity is the amount of enzyme which hydrolyses 1 nmol of substrate/min at 37° C.

b. Percent yield relative to the number of units bound to column.

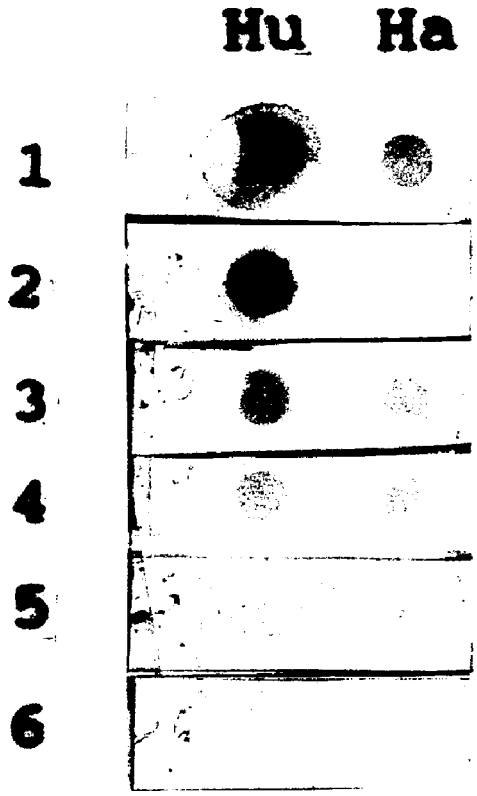


Figure 4.1: Dot immunoblots of human (Hu) and Hamster (Ha) liver α-L fucosidase with anti-human liver α-L-fucosidase polyclonal antibodies (PAb) and monoclonal antibodies (MAb). See Materials and Methods for details.

Column Hu contains 1.5 μg human α-L-fucosidase and column Ha contains 1.5 μg hamster α-L-fucosidase.

Row 1. PAb (10^3 dilution)
Row 2. MAb #116 (10^3 dilution)
Row 3. MAb #100 (10^2 dilution)
Row 4. MAb #72 (10^2 dilution)
Row 5. MAb #40 (10^2 dilution)
Row 6. Secondary antibody (goat-anti-rat IgG/IgM) ($5x10^3$ dilution) (negative control)

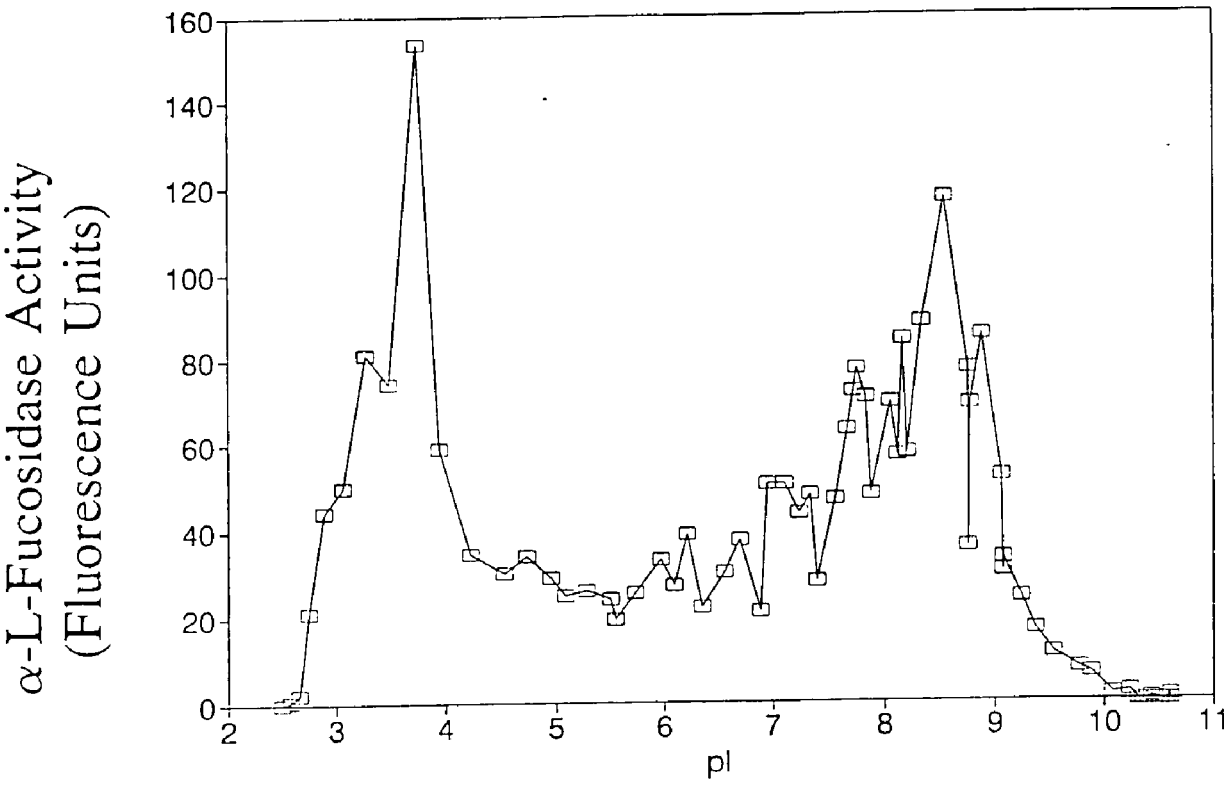


Figure 4.2: Isoelectric focusing profile of purified hamster liver α-L-fucosidase.

hamster liver α-L-fucosidase. Approximately 45% of the activity was associated with several isoforms with pI values above 7. In addition, one to two acidic isoforms between pI values of 3.0-4.0 were found in the purified fucosidase.

SDS-PAGE of 3.0 μg of purified hamster liver α-L-fucosidase (Figure 4.3, lane 2) indicated the presence of a single protein band at 56 kDa compared to two protein bands at 51 and 56 for human liver α-L-fucosidase (lane 1). At a heavier protein loading (9.0 μg) (lane 4), hamster liver fucosidase appears to contain one to two closely-spaced bands between 56 and 60 kDa, and a small amount of what appeared to be a low M_r contaminant at 10 kDa. Western blotting analysis (Figure 4.4) indicated that polyclonal antibodies raised against human liver α-L-fucosidase [19] recognized purified human (lane 2), mouse (lane 1) and hamster (lane 3) liver fucosidase whereas a monoclonal antibody (MAb #116) prepared against human liver fucosidase [21] recognized the human enzyme (lane 5) but not the hamster (lane 6) or the mouse (lane 4) enzymes. The low M_r protein band in the hamster enzyme preparation was not recognized by the polyclonal antibodies, further suggesting that it was a minor contaminant.

The pH activity curve for purified hamster liver fucosidase is shown in Figure 4.5. The curve shows a broad optimum centered around pH 6.8 with relatively high activity between pH 5.5 and 7.5.

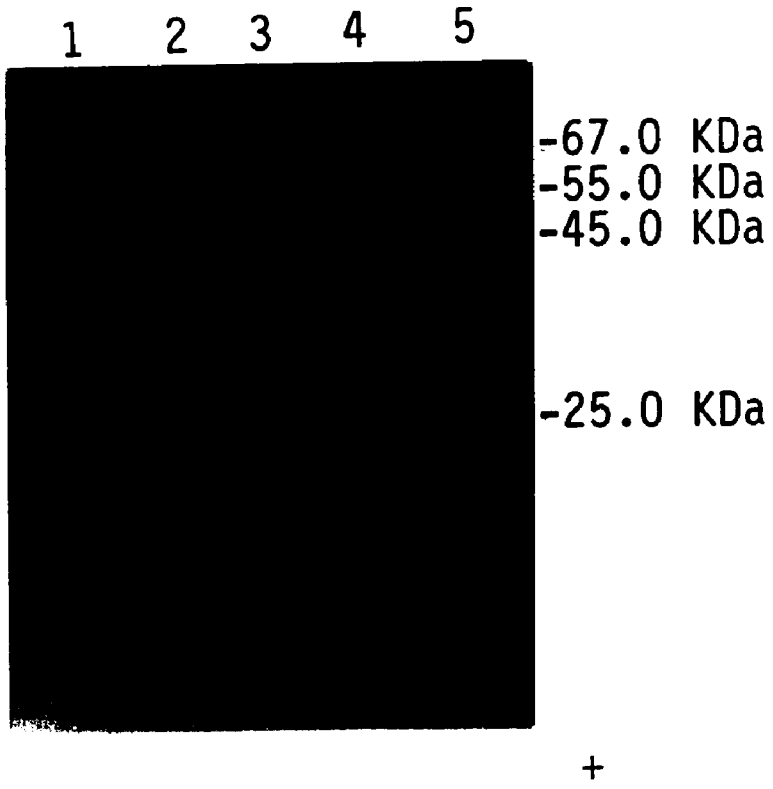


Figure 4.3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified hamster liver α-L-fucosidase. Staining was done with Coomassie Brilliant Blue R-250. The human liver α-L-fucosidase was from a previous study [21].

Lane 1. Human liver α-L-fucosidase (1.5 μg)
Lane 2. Hamster liver α-L-fucosidase (3.0 μg)
Lane 3. Human liver α-L-fucosidase (3.0 μg)
Lane 4. Hamster liver α-L-fucosidase (9 μg)
Lane 5. Molecular weight standards (2.5 μg/standard)

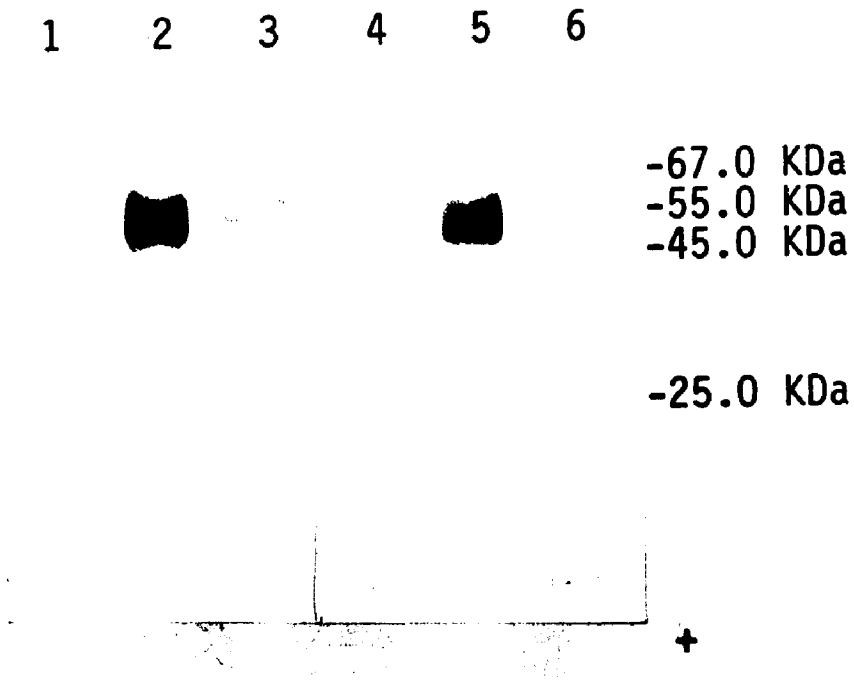


Figure 4.4: Western blotting analysis of purified hamster liver α-L-fucosidase using polyclonal antibodies [19] (lanes 1-3) and a monoclonal antibody (MAb #116) [21] (lanes 4-6) for detection. α-L-fucosidase from mouse liver [22] and human liver [21] were from previous studies.

Lane 1: Mouse liver α-L-fucosidase (4.0 μg).
Lane 2: Human liver α-L-fucosidase (0.5 μg).
Lane 3: Hamster liver α-L-fucosidase (3.0 μg).
Lane 4: Mouse liver α-L-fucosidase (4.0 μg).
Lane 5: Human liver α-L-fucosidase (0.5 μg).
Lane 6: Hamster liver α-L-fucosidase (3.0 μg).

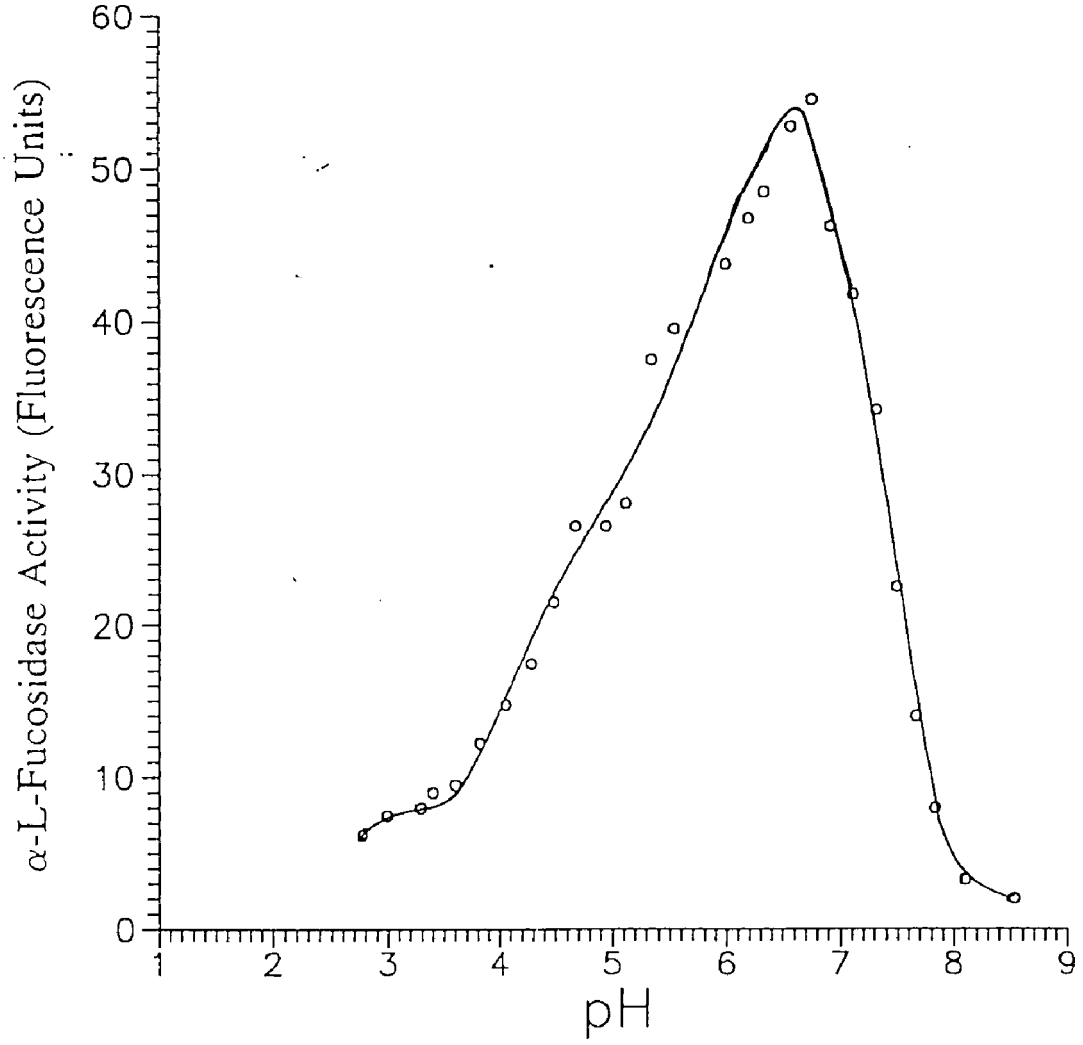


Figure 4.5: pH-Activity curve of purified hamster liver α-L-fucosidase.

4.4. DISCUSSION

In this investigation, the isoform profile of purified liver α-L-fucosidase has been studied. The results indicated that approximately half of the activity of purified hamster liver α-L-fucosidase was associated with isoforms above pI 7.0. However, an unexpected, but reproducible, finding was the presence of one to two acidic isoforms (pI 3.0-4.0) not seen previously in hamster supernatant fluid [3]. α-L-Fucosidase activity was present in hamster liver supernatant fluid at acidic pI values between 3.0 and 5.0 [3], and these acidic isoforms may have been enriched during purification on the affinity resin.

Polyclonal antibodies (PAb's) and four monoclonal antibodies (MAb's) prepared against human liver α-L-fucosidase were used to study the structural relatedness of purified human, mouse and hamster liver α-L-fucosidases. SDS-PAGE results of the hamster enzyme indicated the presence of one to two closely-spaced subunits between 56 and 60 kDa compared to two closely-spaced subunits for the mouse enzyme (57 and 62 kDa) [22], and two for the human enzyme (51 and 56 kDa) [21].

Dot immunoblotting suggested that the MAb's were giving false positive results for hamster fucosidase due to nonspecific interactions with the secondary antibody. Thus, only the PAb's appeared to recognize the hamster enzyme. Western blotting analysis indicated similar results. The hamster and mouse enzymes were recognized by polyclonal

antibodies, but not recognized by a monoclonal antibody (both antibody preparations raised against human liver fucosidase).

The pH optimum of 6.8 for purified hamster liver fucosidase found in the present study was somewhat higher than the optimum of 6.0 found previously for hamster liver fucosidase [3] but this latter value was determined on supernatant preparations and is probably not as reliable.

The overall results (Table 4.2) provide further evidence for some structural differences between human, mouse, and hamster α-L-fucosidases.

TABLE 4.2: SUMMARY OF COMPARISON STUDIES OF HUMAN, MOUSE AND HAMSTER LIVER α-L-FUCOSIDASES.

	Human*	Mouse*	Hamster
Isoelectric focusing studies	4-8 forms pI 4.5-7.5	5-7 forms pI 3-10	4-10 forms pI 3-10
pH optimum (4 Mu fucoside)	pH 4.3, 6.8	pH 5.5	pH 6.8
Binding to agarose-ε-amino caproylfucosamine resin	high	low	intermediate
Subunit analysis	51,56 kDa	57,63 kDa	56,60 kDa

* This information has been provided by Steven Johnson (Chemistry Department).

4.5. REFERENCES

1. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1985) *Biochem. J.* **230**, 75-82.

2. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1987) *Biochem. J.* **245**, 589-593.

3. Johnson, S.W. and Alhadeff, J.A. (1990) *Comp. Biochem. Physiol.* **97B**, 713-717.

4. Alam, T. and Balasubramanian, A.S. (1978) *Biochem. Biophys. Acta* **524**, 373-384.

5. Carlsen, R.B., and Pierce, J.G. (1972) *J. Biol. Chem.* **247**, 23-32.

6. Bosmann, H.B., and Hemsworth, B.A. (1971) *Biochim. Biophys. Acta* **242**, 152-171.

7. Alhadeff, J.A., Miller, A.L., and O'Brien, J.S. (1974) *Anal. Biochem.* **60**, 424-430.

8. Dimatteo, G., Orfee, M.A. and Romeo, G. (1976) *Biochim. Biophys. Acta* **429**, 527-537.

9. Alhadeff, J.A., Miller, A.L., Wenaas, H., Vedvick, T., and O'Brien, J.S. (1975a) *J. Biol. Chem.* **250**, 7106-7113.

10. Robinson, D., and Thorpe, R. (1974) *FEBS Lett.* **45**, 191-193.

11. Alhadeff, J.A., and Janowsky, A.J. (1977) *J. Neurochemistry* **28**, 423-427.

12. Chien, S.F., and Dawson, G. (1980) *Biochim. Biophys. Acta* **614**, 476-488.

13. Opheim, D.J., and Touster, O. (1977) *J. Biol. Chem.* **252**, 739-743.

14. Grove, D.S., and Serif, G.S. (1981) *Biochim. Biophys. Acta* **662**, 246-255.

15. Wright, K., Northcote, D.H., and Davey, R.M. (1976) *Carbohyd. Res.* **47**, 141-150.

16. Laury-Kleintop, L.D. (1986) *Ph.D. Dissertation*, Lehigh University, Bethlehem, Pennsylvania.

17. Fazekas, de St. Groth, S., and Scheideggen, D. (1980) *J. Immunol. Methods* **35**, 1-21.

18. Wewer, V.M., Tichy, D., Damjanov, A., Paulsson, M., and Damjanov, I. (1987) *Dev. Biol.* **121**, 397-407.

19. Andrews-Smith, G.L., and Alhadeff, J.A. (1982) *Biochim. Biophys. Acta* **715**, 753-756.

20. Laemmli, V.K. (1970) *Nature, Lond.* **227**, 680-685.

21. Johnson, S.W., Piesecki, S., Wang, R.F., Damjanov, I., and Alhadeff, J.A. (1992) *Biochem. J.* **282**, 829-834.

22. Shoarinejad, F., Johnson, S.W., and Alhadeff, J.A. (1993) *Comp. Biochem. Physiol.* **105B**, 129-137.

CHAPTER FIVE

PEPTIDE MAPPING AND SEQUENCE ANALYSIS OF THE SUBUNITS AND DEGLYCOSYLATED POLYPEPTIDES OF HUMAN LIVER α-L-FUCOSIDASE

5.1. INTRODUCTION

The structural relationship of the polypeptide chains of the two subunits of human liver α-L-fucosidase is not known. Recent Western blotting studies on human liver α-L-fucosidase have provided evidence for two nonidentical subunits by demonstrating that the 56 and 51 kDa polypeptides are recognized differentially by four monoclonal antibodies and by two lectins, and that two polypeptides are still present after complete removal of carbohydrate with N-glycanase [1]. This latter finding suggests that the two fucosidase subunits are not related solely by glycosylation differences, and that other post-translational modifications and/or polypeptide differences exist. To investigate these possibilities, this chapter describes the use of CNBr peptide mapping performed on glycosylated 56 and 51 kDa subunits and deglycosylated 48 and 45 kDa polypeptides. In addition, the results of amino-terminal sequence analysis of the two subunits are presented.

5.2. MATERIALS AND METHODS

5.2.1. PURIFICATION OF α-L-FUCOSIDASE

α-L-Fucosidase was purified from human liver to apparent homogeneity by affinity chromatography using agarose-ϵ-aminocaproylfucosamine resin (Miles Scientific, Naperville, IL) as described [2]. α-L-Fucosidase activity was assayed using p-nitrophenyl-α-L-fucopyranoside as described [3]. Protein was determined by the method of Lowry et al. [4] using human serum albumin (HSA) as a standard.

5.2.2. SDS-PAGE SEPARATION OF α-L-FUCOSIDASE SUBUNITS FOR PEPTIDE MAPPING

A Bio-Rad Mini-Protean II apparatus (10 sample well, 1.0 mm-thick-slab gels) was used to separate the fucosidase subunits employing the method of Doucet and Trifaro [5]. α-L-Fucosidase samples (10 μg) were incubated for 2 min at 100° C in 70 mM Tris/HCl (pH 6.7) buffer containing 8 M urea, 3% (w/v) SDS, 100 mM dithiothreitol, and 0.005% (w/v) Bromophenol Blue. Samples were electrophoresed in a discontinuous system composed of a 4% stacking gel and a 10% separating gel [100:1 (w/w) acrylamide:bisacrylamide (Bio-Rad, Richmond, CA)] at 155 V for 1 hr in an upper buffer containing 0.1% (w/v) SDS, 100 mM Tris/150 mM glycine (no pH adjustment), and a lower buffer made from a 1:1 (v/v) dilution of upper buffer. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) prepared in methanol/acetic acid/H_2O (2:3:35; v/v/v) for

15 min and destained in methanol/H_2O (1:1; v/v).

5.2.3. N-GLYCANASE TREATMENT OF α-L-FUCOSIDASE

α-L-Fucosidase (10 μg) was denatured by incubation at 100°C for 5 min in 0.5 M Na_2HPO_4 buffer (pH 8.0) containing 0.5% (w/v) SDS and 56 mM β-mercaptoethanol (β-ME). Nonidet P-40 (NP-40) (7.5%, v/v) (Sigma Chemical Co., St. Louis, MO) and recombinant *N*-Glycanase (0.3 units; Genzyme Corp., Farmingham, MA) in 80 mM Na_2HPO_4 (pH 7.5) containing various reagents were added to the denatured fucosidase solution and milli-Q H_2O was added to a final volume of 25 μl. The final concentration of reagents was 0.1% SDS, 1.5% NP-40, 11 mM β-ME, 10% glycerol, 30 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 12 units *N*-Glycanase/mL. All samples were incubated for 24 hr at 37°C. Controls were also run which contained all constituents except N-glycanase. The deglycosylation of *N*-Glycanase-treated α-L-fucosidase was evaluated as previously described [1] by migration changes on SDS-PAGE using 12% gels, and by Western blotting with a digoxigenin-labeled lectin which recognizes both subunits of fucosidase.

5.2.4. CNBr TREATMENT OF FUCOSIDASE SUBUNITS

Subunits were excised individually with a razor blade from the 10% separating gels (100:1 (w/w) acrylamide: bisacrylamide) and each gel slice containing a subunit was cut in half so as to approximate the width of the well for the

subsequent 14% peptide-separating gel (see below). The cut gel slices were placed in 1.5 ml microcentrifuge tubes, and the polypeptides were cleaved *in situ* with CNBr (Sigma, St. Louis, MO) using the general method of Plaxton and Moorhead [6]. The specific conditions which were optimal for CNBr cleavage of α-L-fucosidase were empirically determined after investigating two different acids (70% formic acid & 0.6 N HCl), various incubation times (10 min to 17 h) at 37° C, and differing concentrations of CNBr (20 to 40 mg/4 μg protein). An aliquot (15 μl) of CNBr (100 mg/75 μl acetonitrile) (acetonitrile UV, Baxter, Burdick and Jackson Division, Muskegon, MI) was added to the microcentrifuge tube containing the polypeptide followed by 200 μl of 0.125 M Tris-HCl (pH 6.8) and 200 μl of 0.6 N HCl. CNBr was omitted from controls that contained all other reagents including 15 μl of acetonitrile. Tubes were capped tightly and incubated at 37°C for 30 min with gentle vortexing every 5 min. After incubation, the reaction mixture was removed from the microcentrifuge tube and each gel slice was rinsed with 1.0 ml of 0.125 M Tris/HCl (pH 6.8), containing 0.1% (w/v) SDS and 1 mM EDTA. Each gel slice was then transferred to a 10 mL glass tube containing 3.0 ml of Tris/HCl buffer (pH 6.8) with 0.1% (w/v) SDS and 1 mM EDTA. Tubes were shaken gently at room temperature for 25 min, with three changes of Tris/HCl buffer (pH 6.8) containing 0.1% (w/v) SDS and 1 mM EDTA (the temperature of this solution was approximately 37°C) in order to remove residual CNBr and HCl

and to equilibrate the slice in SDS. All procedures with CNBr were conducted in a fume hood.

5.2.5. SDS-PAGE SEPARATION OF PEPTIDES/GLYCOPEPTIDES

Each CNBr-treated gel slice was transferred to a sample well of a 1.0 mm-thick slab mini-gel prepared as described above except that a 75:2 (w/w) ratio of acrylamide: bisacrylamide was used and the gel consisted of only a 14% (w/v) separating gel [19]. Each gel slice was prodded gently with a Teflon spatula until it rested against the bottom of the sample well. Gels were electrophoresed at 150 V for 1.5 hr and stained with a Bio-Rad silver stain kit (Bio-Rad Laboratories).

5.2.6. DENSITOMETRIC SCANNING OF GELS

Densitometric scanning of gels was performed by using an Ultrascan XL Enhanced Laser Densitometer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) using the method described in the accompanying manual. The extent of CNBr cleavage of fucosidase polypeptides was determined from the densitometric scans by comparing the integrated area for the peak associated with undigested polypeptide to the total integrated area for the peptide map of that polypeptide.

5.2.7. WESTERN BLOTTING OF α-L-FUCOSIDASE AND ITS PEPTIDES/GLYCOPEPTIDES

Following SDS-PAGE separation of the subunits, and the N-Glycanase-treated subunits, the gels were allowed to equilibrate for 30 min in 25 mM Tris/192 mM glycine in 20% (v/v) methanol. The protein was transferred onto 0.2 μm (0.025 μm for peptides/ glycopeptides) nitrocellulose (Schleicher & Schuell, Keene, NH) at 100 V for 1.0 hr (2.0 hr for peptides/glycopeptides) using a Bio-Rad Mini-Trans Blot electrophoresis cell according to the method described in the accompanying manual. A portion of the nitrocellulose was stained for 1 min with 0.1% (w/v) Amido Black 10B (Bio-Rad) in 2-propanol/acetic acid/H_2O (30:10:60; v/v/v) followed by destaining with 2-propanol/acetic acid/H_2O (30:10:60; v/v/v). The remainder of the nitrocellulose was incubated in Tris-buffered saline, TBS (50 mM Tris/HCl, pH 7.5 150 mM NaCl), for 12 hr and then for 1 hr in blocking solution (0.25 g blocking reagent for nucleic acid by hybridization and detection/50 mL TBS, Boehringer Mannheim Biochemicals). Prior to incubation with the lectin-digoxigenin conjugates, the nitrocellulose was washed three times (10 min/wash) in TBS (pH 7.5) and incubated for 10 min in TBS (pH 7.5) containing 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The nitrocellulose was incubated with lectin-digoxigenin conjugates (*Galanthus nivalis agglutinin, GNA*; *Sambucus nigra agglutinin, SNA*; Boehringer Mannheim Biochemicals, Indianapolis, IN) at a concentration of 1 μg

lectin-digoxigenin conjugate/mL of TBS (pH 7.5) containing 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ for 1 hr. The samples were subsequently washed three times (10 min/wash) in TBS followed by a 1 hr incubation with a 10^3 dilution of the Fab fragment of polyclonal sheep anti-digoxigenin coupled to alkaline phosphatase (Boehringer Mannheim Biochemicals). Following three more 10 min washes in TBS, the nitrocellulose strips were placed into 10 mL of color developing buffer [37.5 μL 5-Bromo-4-Chloroindol-3-yl phosphate (BCIP) (50 mg/mL dimethylformamide, DMF) and 50 μL Nitro Blue Tetrazolium, NBT (75 mg/mL in 70% DMF) in 10 mL of 0.1 M, pH 9.5, Tris/HCl containing 50 mM $MgCl_2$ and 0.1 M NaCl] for 2-15 min. The color development reaction was stopped by rinsing the nitrocellulose in milli-Q H_2O.

5.2.8. SEQUENCE ANALYSIS OF POLYPEPTIDES AND CNBr-GENERATED PEPTIDES

The deglycosylated fucosidase polypeptides (48 and 45 kDa) were separated by 10% SDS-PAGE [5] as described above and blotted onto polyvinylidene difluoride (PVDF) membrane (see below) for amino-terminal sequence analysis. CNBr-generated peptides of deglycosylated fucosidase polypeptides were electroeluted from gel slices with a Centrilutor Micro-Electroeluter (Amicon, Inc., Beverly, MA) according to the method described in the accompanying manual. The electroeluted peptides from five preparations were concentrated in

Centricon-3 units (M_r cut-off of 3 kDa) at 7500 x g for 5-6 hr. The peptides were re-electrophoresed at 150 V for 1.5 hr in a 14% (w/v) separating gel (6) as described above, and electroblotted from the gel essentially as described by Reim and Speicher [7]. The gels were soaked in 0.5 X Towbin, Tris-glycine buffer (12.5 mM Tris, 96 mM glycine buffer, 10% (v/v) methanol, pH 8.3) for 30 min to reduce the amount of Tris and glycine. Each gel was sandwiched between a sheet of PVDF membrane (0.2 μm, Bio-Rad) (which had been rinsed with 100% methanol and stored in Tris-glycine buffer for 10 min), and several sheets of filter paper. This was then assembled into a Bio-Rad Mini-Trans Blot electrophoresis cell and electroblotted for 2.5 hr at 2°-4° C and 200 mA (constant current) in Tris-glycine buffer. The PVDF membrane was then washed in milli-Q H_2O for 5 min, stained with 0.025% Coomassie Brilliant Blue R-250 in 40% methanol for 5 min, and destained in 50% methanol, 10% acetic acid for 5-10 min at room temperature. The membrane was finally rinsed in milli-Q H_2O for 5-10 min, air dried and stored at -20° C. The polypeptide and peptide bands were excised from the PVDF membrane and subjected to microsequencing on an Applied Biosystems model 477A sequencer with an on-line 120A PTH analyzer. All sequencing was done at the Macro Core Facility at the Hershey Medical Center (Hershey, PA).

5.3. RESULTS

Human liver α-L-fucosidase was purified 8200-fold with a 74% yield to apparent homogeneity by affinity chromatography using agarose-ε-aminocaproylfucosamine [2]. The final specific activity was 18,100 units/mg protein. This purified fucosidase was used for all the studies described below.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the fucosidase indicated that it was highly purified with two broad protein bands present at 51 and 56 KDa (Figure 5.1[1], lane 2). These two bands have been shown to be immunoreactive with polyclonal antibodies raised against human liver fucosidase, and therefore represent fucosidase protein [1]. The 51 KDa and 56 KDa protein bands, when excised individually from the gel with a razor blade and rerun on SDS-PAGE, migrated as single bands with the appropriate molecular weights (lanes 3 and 4, respectively). Western blotting of the separated 51 KDa and 56 KDa protein bands with digoxigenin-labeled lectins indicated that *Sambucus nigra agglutinin* (*SNA*), a lectin with specificity for α2-6 linked sialic acid residues, recognized the 56 KDa band (lane 6) but not the 51 KDa band (lane 5). *Galanthus nivalis agglutinin* (*GNA*), a lectin with specificity for terminal mannose linked α1-2, α1-3 or α1-6 to mannose, strongly recognized the 51 KDa band (lane 7) and less strongly recognized the 56 KDa band (lane 8). The small amount of *GNA*-positive material in lane

[1]. Figure courtesy of Dr. Susan Piesecki.

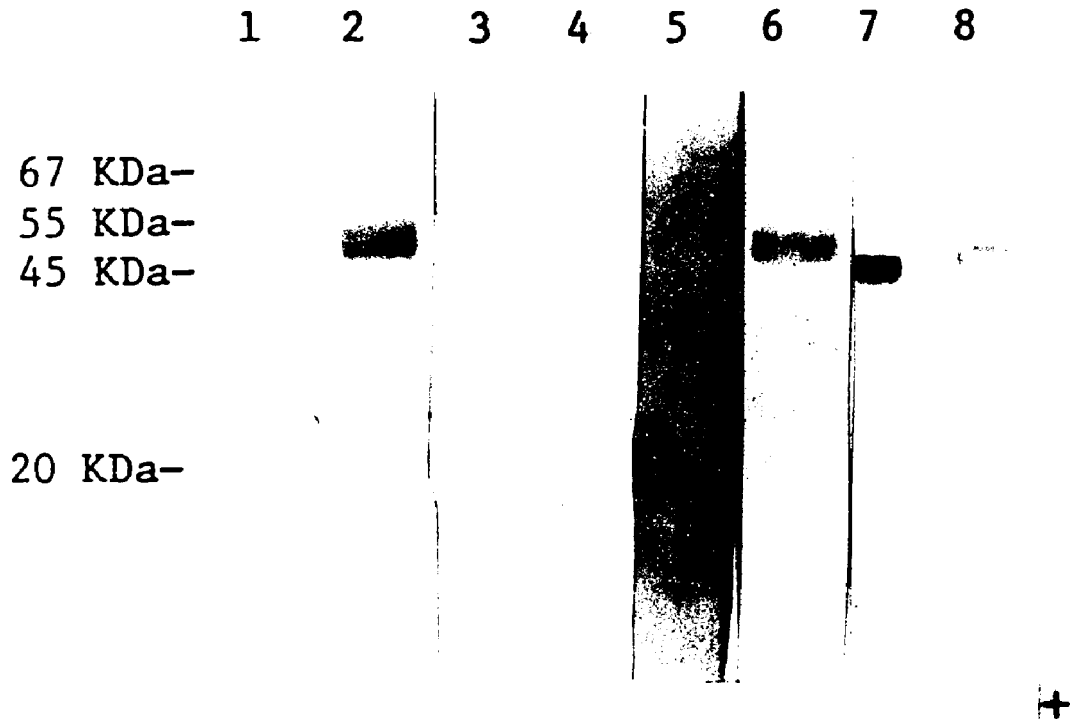


Figure 5.1: Western blot of human liver α-L-fucosidase and its individual subunits. Lanes 1-4 were stained with 0.1% (w/v) Amido Black and lanes 5-8 were stained with lectin-digoxigenin conjugates.

Lane 1. Standard protein M_r markers: 5 μg bovine serum albumin (67 KDa), 10 μg gamma globulin (55 and 20 KDa), and 5 μg ovalbumin (45 KDa)
Lane 2. Human liver α-L-fucosidase (5 μg)
Lane 3. Reelectrophoresis of 51 KDa α-L-fucosidase subunit (2.5 μg)
Lane 4. Reelectrophoresis of 56 KDa α-L-fucosidase subunit (2.5 μg)
Lane 5. Western blot of 51 KDa α-L-fucosidase subunit (2.5 μg) detected with *Sambucus nigra agglutinin* (*SNA*)
Lane 6. Western blot of 56 KDa α-L-fucosidase subunit (2.5 μg) detected with *Sambucus nigra agglutinin* (*SNA*)
Lane 7. Western blot of 51 KDa α-L-fucosidase subunit (2.5 μg) detected with *Galanthus nivalis agglutinin* (*GNA*)
Lane 8. Western blot of 56 KDa α-L-fucosidase subunit (2.5 μg) detected with *Galanthus nivalis agglutinin* (*GNA*)
See Materials and Methods for details.

7 at approximately 25 KDa could be due to very small amounts of a degradation product or a carbohydrate-rich protein contaminant which was not detectable by protein staining (lanes 2 & 3). These data indicate that the two polypeptides are differentially glycosylated and can be separated by SDS-PAGE for *in situ* CNBr peptide mapping.

The results of SDS-PAGE and Western blotting of α-L-fucosidase before, and after *N*-Glycanase treatment are depicted in Figure 5.2[1]. After *N*-Glycanase treatment for 24 hr, a major protein band is seen at 45 KDa and a minor band at 48 KDa (lane 3) rather than the 56 and 51 KDa bands seen prior to *N*-Glycanase treatment (lane 2). Western blotting using digoxigenin-labeled *GNA* indicated the presence of carbohydrate prior to (lane 4), but not after (lane 5), *N*-Glycanase treatment. These data indicate that *N*-Glycanase treatment completely deglycosylates fucosidase, and that the deglycosylated polypeptides also can be prepared by SDS-PAGE for *in situ* CNBr peptide mapping.

Figure 5.3 depicts the SDS-PAGE separation of the peptides/glycopeptides of CNBr-treated glycosylated, and deglycosylated fucosidase polypeptides. Treatment of the glycosylated and deglycosylated subunits (lanes 1 through 4) in control reaction containing all the reagents except CNBr led to no significant breakdown of the polypeptides. CNBr treatment of the 56 and 51 KDa subunits (lanes 5 and 6,

1. Figure courtesy of Dr. Susan Piesecki.

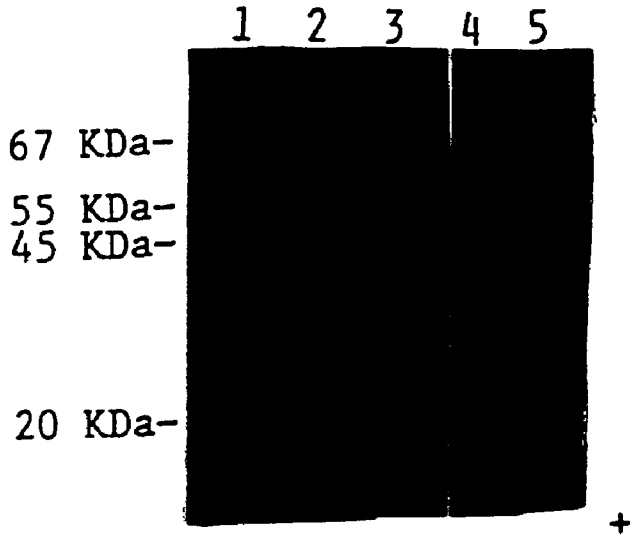


Figure 5.2: SDS-PAGE (lanes 1-3) and western blot (lanes 4-5) of human liver α-L-fucosidase before and after *N*-glycanase treatment.

Lane 1. Standard protein M_r markers: 3 μg bovine serum albumin (67 KDa), 6 μg gamma globulin (55 and 20 KDa) and 3 μg ovalbumin (45 KDa) stained with 0.1% (w/v) Coomassie Blue R-250

Lane 2. Human liver α-L-fucosidase (3 μg) stained with Coomassie Blue R-250

Lane 3. Human liver α-L-fucosidase (3 μg) after 24 h treatment with *N*-Glycanase and stained with Coomassie Blue R-250

Lane 4. Western blot of α-L-fucosidase (10 μg) detected with *Galanthus nivalis agglutinin (GNA)*

Lane 5. Western blot of *N*-Glycanase-treated α-L-fucosidase (10 μg) detected with *Galanthus nivalis agglutinin (GNA)*

Figure 5.3: SDS-PAGE of CNBr-treated glycosylated and deglycosylated human liver α-L-fucosidase subunits.

Lane 1. Glycosylated 56 KDa fucosidase subunit (4 μg) treated with all the peptide mapping reagents except CNBr

Lane 2. Glycosylated 51 KDa fucosidase subunit (4 μg) treated with all the peptide mapping reagents except CNBr

Lane 3. Deglycosylated 48 KDa fucosidase polypeptide (4 μg) treated with all the peptide mapping reagents except CNBr

Lane 4. Deglycosylated 45 KDa fucosidase polypeptide (4 μg) treated with all the peptide mapping reagents except CNBr

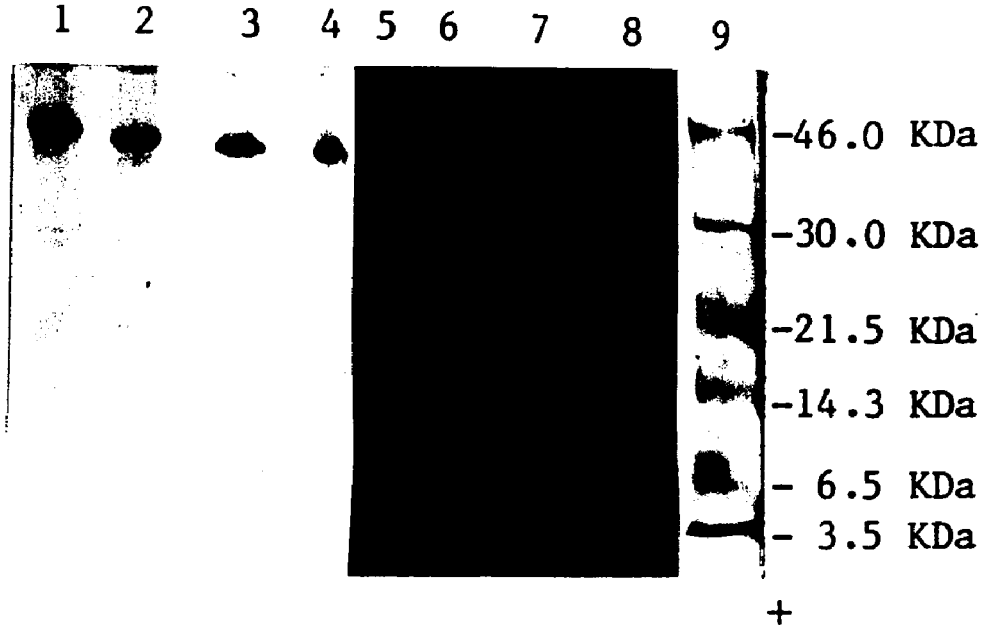
Lane 5. CNBr peptide map of glycosylated 56 KDa fucosidase subunit (2 μg)

Lane 6. CNBr peptide map of glycosylated 51 KDa fucosidase subunit (2 μg)

Lane 7. CNBr peptide map of deglycosylated 48 KDa fucosidase polypeptide (2 μg)

Lane 8. CNBr peptide map of deglycosylated 45 KDa fucosidase polypeptide (2 μg)

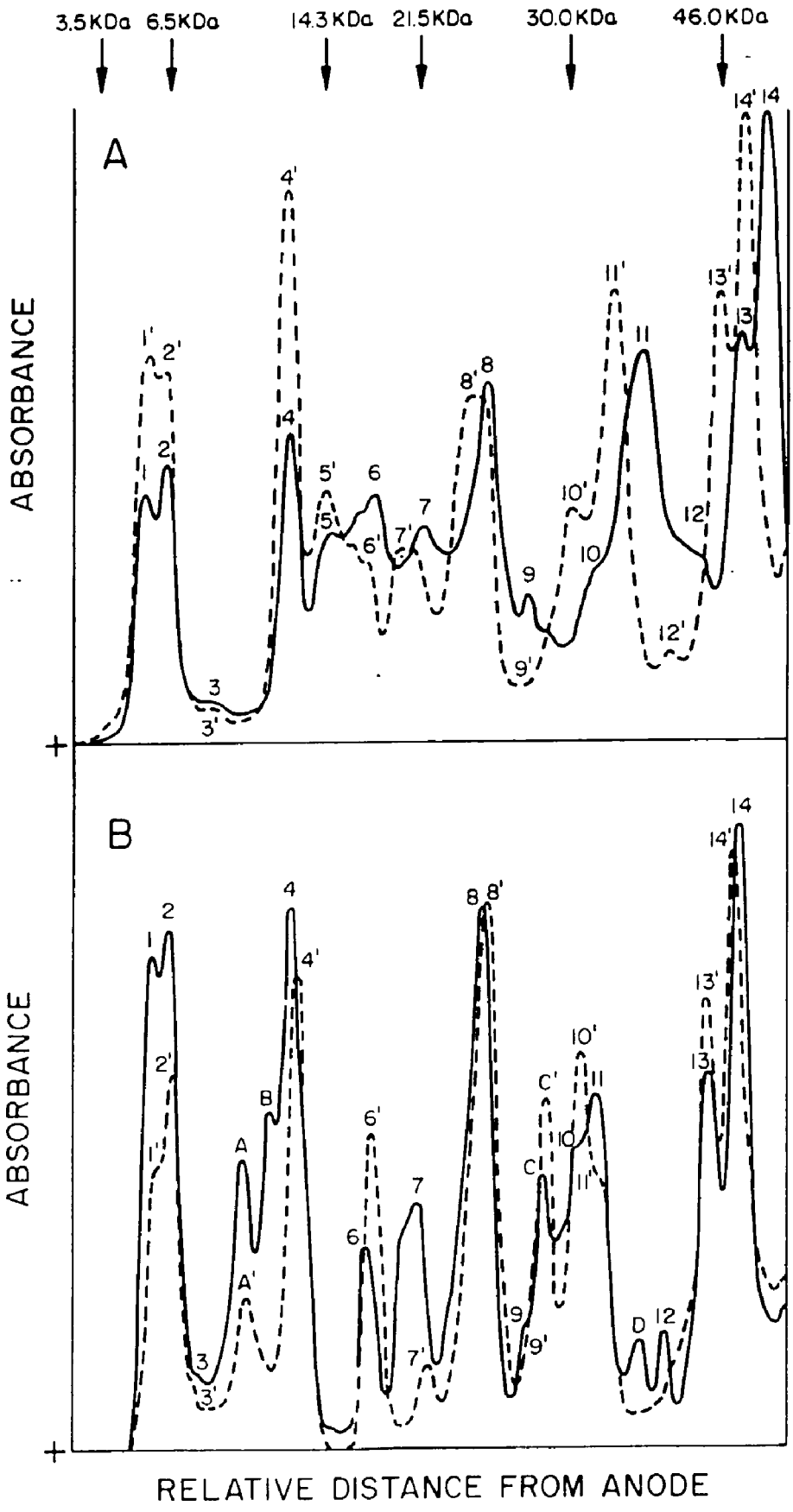
Lane 9. Rainbow molecular weight standards (Amersham Corp, Arlington Heights, IL): ovalbumin (46 KDa), carbonic anhydrase (30 KDa), trypsin inhibitor (21.5 KDA), lysozyme (14.3 KDa), aprotinin (6.5 KDa), insulin b chain (3.5 KDa) stained with a silver stain kit (Bio-Rad). See Materials and Methods for details.
(following page)


1
2
3
4
5
6
7
8
9
-46.0 KDa
-30.0 KDa
-21.5 KDa
-14.3 KDa
- 6.5 KDa
- 3.5 KDa
+

respectively) led to peptide maps with a number of common bands in comparable or differing amount, and a few bands unique to each subunit. The similarities and differences in the banding patterns can be seen more graphically in the densitometric scans of lanes 5 and 6 shown in Figure 5.4, panel A. The peaks are numbered sequentially from the bottom of the gel (anode) using a solid line for the larger subunit and a dashed line for the smaller subunit. Approximate M_rs from the standard proteins run on the SDS gel (Figure 5.3) are indicated at the top of panel A. Several of the 56 KDa subunit peptides (i.e., peaks 7,8,10,11,13,14) are shifted to the right (i.e., have higher M_rs) when compared to the corresponding 51 KDa subunit peptides (i.e., 7′, 8′, 10′, 11′, 13′, 14′).

Peptide maps of the 48 and 45 KDa polypeptides (Figure 5.3, lanes 7 and 8, respectively) resulting from complete deglycosylation with N-Glycanase (see Figure 5.2) led to a loss of peaks 5 and 5′, an elimination of the peak shifts (particularly for the larger peptides), some differences in the relative amounts of the peaks, the appearance of two new peaks (A, A′ and C, C′) in both the larger and smaller polypeptides, as well as the presence of three peaks in the larger polypeptide map (i.e., B, D, 12) that are not detected in the smaller polypeptide map (see Figure 5.4, panel B). The peak shifts of the larger subunit seen in panel A (e.g., 7, 11, & 14) are toward the lower M_r peaks of the smaller subunit

Figure 5.4: Densitometric scans of CNBr peptide maps of glycosylated (panel A) and deglycosylated (panel B) fucosidase subunits (from Figure 5.3, lanes 5-8). Solid lines represent 56 KDa subunit (panel A) and 48 KDa polypeptide (panel B), and dashed lines represent 51 KDa subunit (panel A) and 45 KDa polypeptide (panel B). Molecular weights of protein markers are indicated across the top of panel A. (following page)


3.5KDa
6.5KDa
14.3KDa
21.5KDa
30.0KDa
46.0KDa
A
B
ABSORBANCE
ABSORBANCE
+
+
RELATIVE DISTANCE FROM ANODE

in the scans of the deglycosylated subunits (panel B). This suggests that the original shifts in panel A were due to carbohydrate differences.

The extent of CNBr cleavage of each fucosidase polypeptide was determined from the densitometric scans (Figure 5.4, panels A and B) to be approximately 88-90% by comparing the integrated area for the peak associated with undigested fucosidase (peak 14 or 14′) to the total integrated area for the peptide map of that polypeptide.

Western blotting of the peptides/glycopeptides produced from CNBr treatment of the 56 and 51 KDa glycosylated subunits of fucosidase provided further evidence that the shifts seen in the densitometric scans described above (Figure 5.4, panel A) were due, at least in part, to carbohydrate differences. Figure 5.5 indicates that the 51 KDa (lane 1) and 56 KDa (lane 2) subunits contained seven to eight *GNA*-positive bands with M_rs from approximately 14 to 50 KDa. The 56 KDa subunit (lane 2) contained minor *GNA*-positive bands at approximately 20 and 28 KDa which were barely detectable in the 51 KDa subunit (lane 1). The major *GNA*-positive bands at or above 30 KDa appear to correspond to the high-M_r shifted peaks (e.g., 11 & 11′, 13 & 13′, 14 & 14′) seen in Figure 5.4, panel A. No *GNA*-positive bands were seen for either subunit at M_r values below 14 KDa, consistent with the superimposable peaks (e.g., 1 & 1′, 2 & 2′, 3 & 3′, 4 & 4′, 5 & 5′) found at low M_rs for the 56 and 51 KDa subunits (Figure 5.4, panel A).

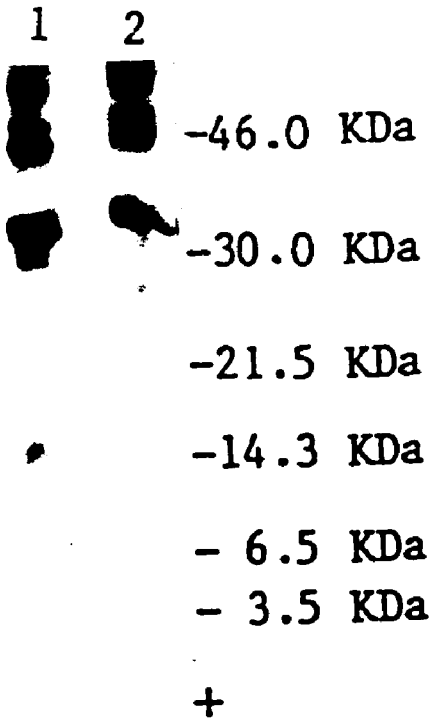


Figure 5.5: Western Blot of Peptides and Glycopeptides Produced from CNBr Treatment of the Glycosylated Subunits of α-L-Fucosidase using the Lectin *Galanthus nivalis agglutinin (GNA)*.

Lane 1. Glycopeptides from 51 KDa subunit
Lane 2. Glycopeptides from 56 KDa subunit
See Materials and Methods for details.

Sequence analysis of the 48 and 45 kDa deglycosylated fucosidase polypeptides yielded no amino-terminal amino acidresidues, suggesting that the amino-termini of both polypeptides were blocked. Therefore, peptide fragments generated from CNBr treatment of the 48 and 45 kDa polypeptides were examined. The largest CNBr peptide of each polypeptide (bands corresponding to peaks 14 & 14' of Figure 5.4, panel B) was sequenced, and the results are shown in Table 5.1. These results indicated an identical 13 amino acid sequence (Pro Pro Arg Arg Tyr Thr Pro Asp Trp Pro Ser Leu Asp). This corresponds to a sequence beginning at amino acid six of the sequence deduced from the cDNA for human liver α-L-fucosidase. The amino acid residue at position 19 of the lower M_r subunit is Ser. However, a clear determination of the corresponding amino acid in the higher M_r subunit could not be established. Analysis of three other CNBr peptides from each polypeptide (bands corresponding to peaks 13 & 13' 11 & 11', 8 & 8', of Figure 5.4, panel B) yielded no amino-terminal amino acid residues. The failure to generate a sequence for these peptides may be explained by the chemical peculiarities of the peptide fragments or insufficient amounts of material available for analysis. Taken together, these results suggest that the amino-terminal regions of both the 48- and 45-KDa proteins are very similar and possibly even identical. These results do not rule out the possibility that the C-terminal regions of these polypeptides may differ.

TABLE 5.1: AMINO-TERMINAL SEQUENCES OF CNBr-GENERATED PEPTIDE FRAGMENTS OF THE DEGLYCOSYLATED HUMAN LIVER α-L-FUCOSIDASE SUBUNITS

PEPTIDE	SIZE (kDa)	PEAK[a] NO.	N-TERMINAL SEQUENCE AT RESIDUE POSITION[b]													
			6	7	8	9	10	11	12	13	14	15	16	17	18	19
High M_r subunit	~ 48	14	Pro	Pro	Arg	Arg	Tyr	Thr	Pro	Asp	Trp	Pro	Ser	Leu	Asp	
Low M_r subunit	~ 45	14	Pro	Pro	Arg	Arg	Tyr	Thr	Pro	Asp	Trp	Pro	Ser	Leu	Asp	Ser

a. Peak numbers refer to figure 5.4.
b. Numbers correspond to the numbers of amino acid residues which are deduced from nucleotide sequencing of the cloned gene.

5.4. DISCUSSION

Previous Western blotting studies using lectins have shown that the two subunits of human liver α-L-fucosidase differ with regard to glycosylation [16]. However, two polypeptides remained after complete removal of carbohydrate with N-Glycanase, suggesting that fucosidase contains two different polypeptides. In the present investigation further evidence for differences in the polypeptide chains of α-L-fucosidase has been provided by CNBr peptide mapping of the enzyme's glycosylated and deglycosylated subunits. The subunits and deglycosylated polypeptides were separated by SDS-PAGE, excised, and subjected to *in situ* cleavage with CNBr using standard procedures which have provided useful structural information to several investigators who have used CNBr [19-22] or acid [23-25] treatments for *in situ* digestions. Our CNBr treatment cleaved approximately 90% of the intact fucosidase polypeptides. A larger number of peptides were found than the six predicted due to the presence of five Met residues deduced from the cDNA of at least one of the fucosidase subunits [10]. The presence of a larger than predicted number of CNBr peptides has been found in other studies, and has usually been attributed to incomplete digestion of large peptides [19-21]. The larger than expected number of peptides in our study also probably resulted from incomplete digestion of large peptides due to the short incubation time used. Therefore, some of the larger M_r

peptides must contain one or more Met residues. The short incubation time was determined empirically and chosen as a compromise to maximize CNBr cleavage and to minimize nonspecific acid degradation of the fucosidase polypeptides. At any rate, identical conditions were used for treating both subunits and both deglycosylated polypeptides so that a comparative analysis could be done.

The densitometric scans of the peptide maps of the glycosylated 56 and 51 kDa subunits were similar, but the larger subunit had several bands shifted to higher apparent M_rs when compared to the smaller subunit. These shifts disappeared when *in situ* CNBr peptide mapping was performed on the polypeptides after complete deglycosylation by N-Glycanase, suggesting that the original shifts were due to carbohydrate differences. Further evidence that the peak shifts were due to carbohydrate differences was provided when Western blotting of the peptides and glycopeptides of the glycosylated subunit indicated that only peptides with M_rs > 14 kDa were *GNA*-positive. Several differences not related to glycosylation were found between the deglycosylated polypeptides, including the presence of three peptides in the larger polypeptide not detected in the smaller polypeptide. These results provide the first direct evidence that polypeptide chain differences exist between the 56 and 51 kDa subunits in addition to the glycosylation differences demonstrated previously [1], and confirmed in the present

investigation. It is possible that other post-translational modifications account for the differences between the deglycosylated 48 and 45 kDa polypeptides but this is less likely than polypeptide chain differences.

Amino-terminal sequence analysis of the two deglycosylated fucosidase polypeptides did not yield any amino acid residues. This suggests that the amino-termini of both subunits are blocked. Sequence analysis was performed on corresponding CNBr-generated peptides from each polypeptide. This analysis indicated an identical sequence of 13 amino acids on the largest CNBr fragment of each polypeptide, which corresponds to residues 6 through 18 of the sequence deduced from the cDNA for human fucosidase [8].

The overall results indicate that the deglycosylated polypeptides of human liver α-L-fucosidase are very similar but probably not identical. The great similarity suggests that both polypeptides are products of the same gene, and that the larger polypeptide may be the precursor to the smaller polypeptide. The minor differences in the polypeptides, other than those due to glycosylation which were demonstrated previously [1] and further confirmed in the present study, could also be due to post-translational modifications.

5.5. REFERENCES

1. Johnson, S.W., Piesecki, S., Wang, R.F., Damjanov, I., and Alhadeff, J.A. (1992) *Biochem. J.* **282**, 829-834.

2. Alhadeff, J.A., Miller, A.L.; Wenaas, H., Vedvick, T., and O'Brien, J.S. (1975) *J. Biol. Chem.* **250**, 7106-7113.

3. Alhadeff, J.A., and O'Brien, J.S. (1977) in *Practical Enzymology of the SphingoLipidoses* (Glew, R.H., and Peters, S.P., Eds) Alan R. Liss, Inc., New York, pp. 247-281.

4. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275.

5. Doucet, J.P., and Trifaro, J.M. (1988) *Anal. Biochem.* **168**, 265-271.

6. Plaxton, W.C., and Moorhead, G.B.G. (1989) *Anal. Biochem.* **178**, 391-393.

7. Reim, D.F., and Speicher, D.W. (1992) *Anal. Biochem.* **207**, 19-23.

8. Ochiodoro, T., Beckman, K.R., Morris, C.P., and Hopwood, J.J. (1989) *Biochem. Biophys. Res. Commun.* **164**, 439-445.

CHAPTER SIX

SUMMARY

6.1. SUMMARY

Preliminary studies on supernatant fluids have shown that mouse and hamster liver α-L-fucosidase may be different from other mammalian α-L-fucosidases. The isoform profiles of α-L-fucosidase from rodent (mouse, guinea-pig, rat, hamster) spleen, brain and liver tissues were studied. The results suggest that fucosidase isoform above pI 7.0 are present only in mouse and hamster spleen, brain and liver. It thus appears from the present and past studies [1-3,5] that, of the rodents studied, only mouse and hamster contain the atypical α-L-fucosidase with significant amounts of isoforms above pI 7.0 (summarized in Table 2.2). The differences in the isoelectric focusing profiles of these two α-L-fucosidases compared to that of the human, guinea-pig, and rat liver α-L-fucosidase is likely to be the result of differences in their amino acid compositions. The relative amounts of charged residues effect the isoelectric focusing profiles. This atypical fucosidase, may represent a variant form of the enzyme with an, as yet, unknown specialized function.

Western blotting analysis was performed on the subunits of mouse α-L-fucosidase using lectins, polyclonal antibodies (PAbs) [6] and monoclonal antibodies (MAbs) [7] prepared against human liver α-L-fucosidase, and the results were compared to the results of similar studies on human liver α-L-fucosidase [7]. These studies indicated that both of the mouse subunits were immuno-reactive with antibodies raised against

human α-L-fucosidases. This suggests that the high-M_r subunit (62 kDa) is not unique and shares some sequence identity with at least one of the human subunits. Lectin analysis indicated that both mouse subunits are *GNA*-positive and *SNA*-positive. This differs from human fucosidase in which the low-M_r human subunit is positive only with *GNA* whereas the high-M_r human subunit is positive with both *GNA* and *SNA* [7]. This suggests that both mouse subunits contain sialic acid residues. This gives further evidence that although human and mouse liver α-L-fucosidase share some epitopes, differences in their antigenic and structural properties can be observed with monoclonal antibodies and lectins.

Since mouse liver α-L-fucosidase has already been purified and characterized [4,8], the purification of hamster liver α-L-fucosidase was undertaken so that its properties could be compared to those of human and mouse α-L-fucosidases. Isoelectric focusing of purified hamster liver α-L-fucosidase indicated that this enzyme contains approximately half of its activity associated with isoforms above pI 7.0. SDS-PAGE indicated that the purified enzyme consists of one to two closely-spaced protein bands (subunits). Western blotting analysis indicated that the hamster enzyme is recognized by polyclonal goat-anti-human liver α-L-fucosidase antibody but not recognized by a monoclonal antibody prepared against human liver α-L-fucosidase. The pH optimum of purified hamster liver α-L-fucosidase is somewhat higher than the optimum found

previously for hamster liver supernatant. These studies give further evidence that although human, mouse and hamster liver α-L-fucosidases share some epitopes, differences in their structures can be observed with a monoclonal antibody.

Recent Western blotting studies on human liver α-L-fucosidase have provided evidence for two non-identical subunits by demonstrating that the high-M_r subunit and the low-M_r subunit are recognized differentially by four monoclonal antibodies and by two lectins, and that two polypeptides (subunits) are still present after complete removal of carbohydrate with N-glycanase [7]. This finding suggests that the two subunits are not related solely by glycosylation differences and that other post-translational modifications and/or polypeptide differences exist. Further evidence for some structural differences and/or similarities existing between the two human liver α-L-fucosidase subunits was provided by CNBr peptide mapping and amino terminal sequence analysis. The two human liver α-L-fucosidase subunits yielded very similar peptide maps, but the larger subunit had several high-M_r bands shifted to higher apparent molecular weights when compared to the smaller subunit. The deglycosylation of the polypeptides by N-glycanase resulted in the disappearance of these shifts. However, minor differences not related to glycosylation were still present between the deglycosylated polypeptides. Jill Zhu [9] documented similar results in the tryptic peptide mapping studies. These studies

indicated that 6 of the 30 tryptic peptides found in the larger deglycosylated subunit (48 kDa) were not seen in the smaller deglycosylated polypeptide (45 kDa). Amino-terminal sequence analysis of the largest CNBr-generated peptides from the two deglycosylated polypeptides indicated an identical sequence of 13 amino acids on each fragment. Taken together, the results of peptide mappings and N-terminal sequence analysis indicate that the deglycosylated polypeptides of human liver α-L-fucosidase are very similar but not identical. The great similarity suggests that both polypeptides are products of the same gene, and that the larger polypeptide may be the precursor to the smaller polypeptide. The minor differences in the deglycosylated polypeptides could also be due to post-translational modifications such as hydroxylations, phosphorylations, acetylations, methylations, nucleotidylations, or other "miscellaneous" modifications.

The obvious extension of sequence studies detailed in this dissertation is to repeat N-terminal sequencing of CNBr-treated peptides. Several peptides in higher quantity must be prepared and subjected to sequencing. Sequencing of peptides which exist in one polypeptide, but not other, would yield information on any possible sequence differences between the two subunits. However, the direct determination of the entire amino acid sequence to pin the difference is a major undertaking. On the other hand, chemical peculiarities of individual peptide fragments might prevent the process from

being routine.

Several cDNAs encoding human fucosidase have been cloned and sequenced, and only one gene coding for active fucosidase have been so far mapped. Thus, it appears, up to now, that there exists a single gene that codes for active α-L-fucosidase. However, there might be alteration in the pattern of RNA splicing and consequently production of more than one polypeptide. This fact points to the importance of RNA studies, since the presence of two subunits might be a result of different processing of α-L-fucosidase mRNA.

The knowledge concerning the molecular biology of the α-L-fucosidase gene is somewhat uncertain. Examining this aspect of fucosidase in both the normal and diseased state would allow the characterization of fucosidosis at the molecular level.

6.2. REFERENCES

1. Johnson, S.W., and Alhadeff, J.A. (1990) *Comp. Biochem. Physiol.* **97B**, 713-717.

2. Laury-Kleintop, L.D., Alhadeff, J.A., and Damjanov, I. (1985) *Dev. Biol.* **111**, 520-524.

3. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1985) *Biochem. J.* **230**, 75-82.

4. Laury-Kleintop, L.D., Damjanov, I., and Alhadeff, J.A. (1987) *Biochem. J.* **245**, 589-593.

5. Shoarinejad, F., Johnson, S.W., and Alhadeff, J.A.(1993) *Comp. Biochem. Physiol.* **105B**, 129-137.

6. Andrews-Smith, G.L., and Alhadeff, J.A. (1982) *Biochim. Biophys. Acta* **715**, 90-96.

7. Johnson, S.W., Piesecki, S., Wang, R.F., Damjanov, I., and Alhadeff, J.A. (1992) *Biochem. J.* **282**, 829-834.

8. Shoarinejad, F., Johnson, S.W., and Alhadeff, J.A. (1993) *Comp. Biochem. Physiol.* **105B**, 129-137.

9. Shoarinejad, F., Zhu, J., Bazel, S.B., and Alhadeff, J.A. (1994) (submitted).

PUBLICATIONS

1. Shoarinejad, F., Johnson, S.W., and Alhadeff, J.A. (1993) "Analysis of the subunit, isoforms, and substrate specificity of mouse liver α-L-fucosidase." *Compar. Biochem. Physiol.* **105B**, 129-137.

2. Shoarinejad, F., and Alhadeff, J.A. (1993) "Rodent tissue α-L-fucosidase: Analysis of brain and spleen isoforms and characterization of the purified hamster liver enzyme." *Compar. Biochem. Physiol.* **105B**, 523-528.

3. Shoarinejad, F., Zhu, J., Bazel, S.B., and Alhadeff, J.A. (1994) "Peptide mapping and sequence analysis of the subunits and deglycosylated polypeptides of human liver α-L-fucosidase." *Arch. Biochem. Biophys.* **312**, No.1, 173-179.

4. Bazel, S.B., Ferry, K., Shoarinejad, F., Laury-Kleintop, L., Lang, M.K., Tachovsky, T., Longo, S., Tucker, S., and Alhadeff, J.A. (1994) "Analysis of breast tissue cathepsin D isoforms from patients with breast cancer, benign breast disease and from normal controls." Submitted.

VITA

Fariba Shoarinejad was born to Ali Akbar Shoarinejad and Fatemeh Alizadeh Ashrafi on October 12, 1959 in the northeastern city of Tabriz in Iran. She graduated from Tehran University with a bachelor of science degree in chemistry in the spring of 1986. Fariba moved to the United States of America to further her education in the Spring of 1988. She attended Lehigh University in Bethlehem, PA in Spring of 1991 and worked under the direction of Professor Jack A. Alhadeff on the comparative characterization of rodent and human α-L-fucosidase. Fariba recieved a M.S. in chemistry in October of 1993 and remained in Professor Alhadeff's laboratory to pursue a Ph.D. in biochemistry.