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AFFINITY CHROMATOGRAPHY OF RABBIT GASTROCNEMIUS ALPHA-L-FUCOSIDASE

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Abstract: Ct-L-Fucosidase was purified from rabbit gastrocnemius muscle using agarose-e-aminocaproyl-fucosamine. The isolated enzyme was found to be pure by a number of different analytical techniques including gel electrophoresis. The purified enzyme was found essentially free of other glycosidases. The main characteristics of the purified α-L-Fucosidase were determined; pH 6.0-6. 3, KmO.52, Vmax 25.13 n mol/gm protein/min., Ki 0.46. L-fucosq was the only physiological competitive inhibitor of the enzyme. Ct-L-fucosidase is heat labile and stable for 2-3 months at — 20 C in the presence of lOOmM L-fucose. The purified enzyme showed 50,000 Mol. Wt. on SDS-Electrophoresis.

Introduction

Alpha-L-Fucosidase is an important enzyme in the metabolism of biological substances containing L-fucose.[1] α-L-Fucosidase is essential for the further study of genetic disorder of fucosidosis in which this enzyme is defective, and for any proposed attempts at enzyme replacement in these cases. α-L-Fucosidase is also very important for the studying the role of fucose residues in the lysosomal membrane. The fucose residues have been implicated for binding of specific proteins[2] and possibly determine intracellular transport. The affinity method reported earlier by us[3] and in this study is very useful for the characterization of α-L-fucosidase. The affinity method might also be applicable to other hydrolases.

Experimental Procedures

Materials

Agarose-e-aminoeaproyl-fucosamine was purchased from Miles Laboratories Limited, Stoke Poges, Slough, England. 4-methyumbelliferyl-α-L- fucopyranoside; L-fucose; Acrylamide; Tris (2-amino-2) (hydroxymethyl)-l, 2 propanediol; Bis (N, N'-methylene-diacrylamide) were purchased from Koch-Light Laboratories, Colnbrook, Buckinghamshire, England. Bovine serum albumin; SDS-Molecular weight markers were purchased from Sigma

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Chemical Co., St. Louis, Missouri, U.S.A. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals employed were of suitable analytical grade. Adult male wistar rats were used.

Enzyme Preparation

The animals were killed by cervical dislocation. Gastrocnemius muscles were removed from the hind legs and quickly rinsed in ice-cold isotonic medium (0.25 M sucrose in 5mM tris-HCl buffer, pH 7.4). All procedures were carried out at 0-4°C.

Preparation of Sample

A 20% (w/v) homogenate of gastrocnemius muscle was prepared in l0mM sodium phosphate buffer, pH 5.5, containing 1mM EDTA. The homogenate was sonicated for 60s and following stablisation at 4°C for 2-3 h, was centrifuged at 70,000 g for 20 min. The supernatant (S1) was kept and the pellet obtained was raised to the minimum volume (5-10 ml) in the original buffer. The pellet suspension was resonicated and centrifuged as before. The supernatant (S2) obtained was pooled with SI and the pellet discarded.

Ammonium Sulphate Fractionation

The pooled supernatant (S1+S2) was brought to 30% saturation with solid ammonium sulphate. The mixture was centrifuged at 40,000 g for 15 min. The pellet obtained was raised to a minimum volume in the same buffer and the supernatant was brought to final saturation of 65% with $(NH_4)_2S0_4$. The dissolved pellet was centrifuged at 40,000 g for 20 min. The supernatant was discarded and the pellet was dissolved in 10 ml of 50mM sodium phosphate buffer, pH 6.8.

Desalting

A column (3 x 25 cm) was packed with Sephadex G-25 and equilibrated at 4° C with several column volumes of 50mM sodium phosphate buffer, pH 6.8. The dissolved pellet (30-65%) $(NH_4)_2S0_4$ was desalted by passing through this column. After discarding the void volume (60 ml) 5 ml fractions were collected on a LKB fraction collector.

The enzymatically active fractions were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane. The concentrated sample was dialysed for 20 h with two changes against 6 liters of original buffer. The dialysate was then centrifuged at 70,000 g for 30 min. The pellet was discarded and the supernatant (S3) was retained for affinity chromatography.

Affinity Chromatography

A column (0.6 x 10 cm) was packed with Agarose-amino caproyl-fucopyranosylamine and equilibrated with 50mM sodium phosphate buffer, pH 6.8. The enzyme sample (S3) was applied onto the column and a flow rate of about 15 ml/h was maintained. The column was washed with the equilibrating solution at the same rate until the absence of absorbance at 280 nm in the eluate. α-L-Fucosidase was eluted with a 1% (W/V) solution of L- fucose (25 ml) in the same buffer solution.

Fractions of 2 ml were collected and enzymatically active fractions were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane. The concentrated sample was dialysed for 28 h with four changes against 6 litres of original buffer. Dialysis at this stage constitutes an essential step since L-fucose has an inhibitory effect on α-L-fucosidase. The dialysate was centrifuged at 70,000 g for 30 min. The pellet was discarded and the supernatant was retained for the characterisation of α-L-fucosidase. To prepare the column for reuse, it was washed with 2 M NaCl in the same buffer (50 ml) and equilibrated with the original buffer.

Polyacrylamide Gel Electrophoresis of the Purified Enzyme

Polyarcrylamide gel electrophoresis of the purified enzyme was performed on rod gels of 7% acrylamide according to the method of Davis[4] with the modification in the pH of buffer used. Since the enzyme was found to be highly unstable above pH 8.5, the gel was polymerised at pH 7.5 and electrophoresis was done in 0.025 M Tris (0.192 M) glycine buffer, pH 7.4 at 2mA/tube for 4-6 h until the marker dye band was within 5 mm of the bottom. After the electrophoresis each gel was cut into two longitudinal halves. One half was transversely sliced into 5mm pieces and each piece was homogenised in 0.2mM citrate phosphate buffer containing 1.25mM 4- methylumbeliferyl α-L-fucopyranoside. Each homogenate was then incubated at 37°C for 30 min and 3 ml of cold glycine buffer, pH 10.7, was added. The mixture was centrifuged at 1000 g for 5 min and the fluorescence of the supernatant was measured. The other half was stained for protein by coomassie Brilliant Blue R.

Enzyme Assay

The assay mixture contained 50 ul of the enzyme sample, 100 ul of 0.3 M citrate-phosphate buffer and 100 ul of the flourimetric substrate (1.25 mM). Assay mixture was incubated at 37°C for 20 min in an oscillating water bath and the reaction was stopped by the addition of 3 ml of ice-cold glycine buffer (0.2 M), pH 10.7, containing 0.1 M Na_2C0_3 and 0.125M NaCl. The liberated aglycone was measured using Zeiss SFM4C Spectro- flourimeter at a 365nm exciting, and 448 nm emitting, wavelengths. Enzyme activity was expressed as nanomoles of 4-methylumbelliferone released per minute and specific activity as nanomoles of 4-methylumbelliferone released per mg protein per min.

Kinetic Studies

Apparent Mischaelis constants (Km values) and maximal velocities (Vmax) were determined for purified a-L-fucosidase using 4-methylumbelli- feryl substrate. Enzyme sample was incubated at 37°C for 20 min in quadruplicate using (100 ul) of varying concentrations of substrate in 100 ul of citrate-phosphate buffer at the optimum pH of the enzyme. Kinetic parameters were calculated using an ICL 190S computer and a programme (entitled POLY) written in FORTRAN.

Ki (inhibition constant) was determined by mixing the purified preparation of α-L-fucosidase with varying concentrations of L-fucose and assaying under standard conditions.

Results

The purification of α-L-fucosidase on ACF-sepharose resulted in a relatively good yield of activity. The pooled supernatant was applied onto ACF-sepharose column and the elution pattern is shown in Fig-1. A summary of the purification steps of α-L-fucosidase is presented in Table-1. The enzyme was purified 604-fold to a specific activity of about 43 n mol per mg protein per min. A total yield of 30% was achieved.

Assay of Purified a-L-Fucosidase for Contaminating Glycosidase

The purified preparation of a-L-fucosidase from rabbit gastrocnemius muscle was found to have only trace amount of other glycosidase activities as shown in Table-2. Of all the glycosidases studied N-acetyl-β-D-glucosaminidase exhibited maximum of 0.78% of the fucosidase activity.

Kinetic and Inhibition Studies

Apparent Michaelis constants (Km) for purified a-L-fucosidase from gastrocnemius muscle of rabbit was determined under conditions described in methods. Double reciprocal plots, based on the linear regression analysis of Lineweaver-Burk,[5] were found to be linear for the purified enzyme. The data were analysed by the use of a computer programme which was specifically designed to determine apparent Km values and Vmax values. The results obtained are presented in Table-3. The apparent Km value obtained for purified enzyme of gastrocnemius muscle was not significantly different although Vmax values varied considerably.

L-Fucose is the only potential physiological effector of α-L-fucosidase to have been identified.[2] The enzyme was competitively inhibited by L- fucose as determined by Lineweaver Burk plot. The Ki and Km values obtained are presented in Table-3. The inhibition constant (Ki) 0.46 was obtained and Km 0.052 with Vmax 25.13 was determined. Certain divalent ions, $Cd^{2}+$, $Cu^{2}+$, $Hg^{2}+$, $Ag^{2}+$, had inhibitory effect causing almost a total inhibition at a concentration of 2.5 mM.

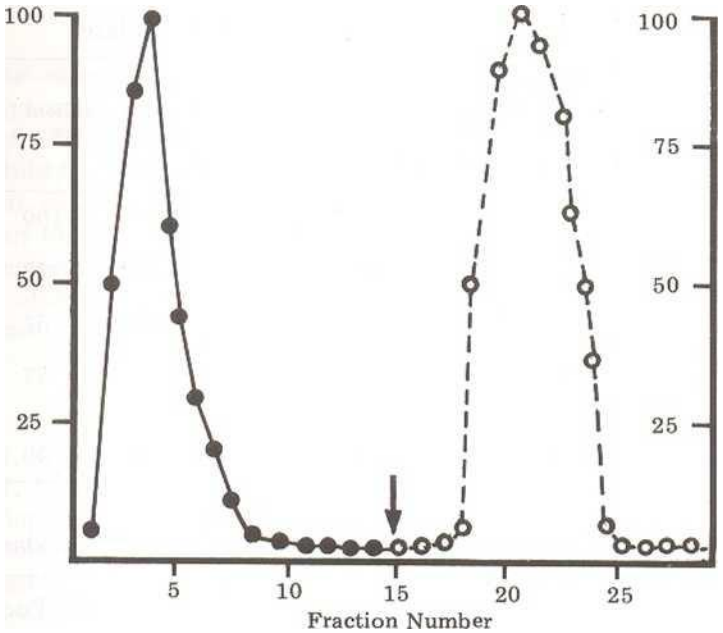

Fig. 1 Affinity Chromatography of rabbit Gastrocnemius muscle α-L-fucosidase on ACF-sepharose. Application of L-fucosidase.

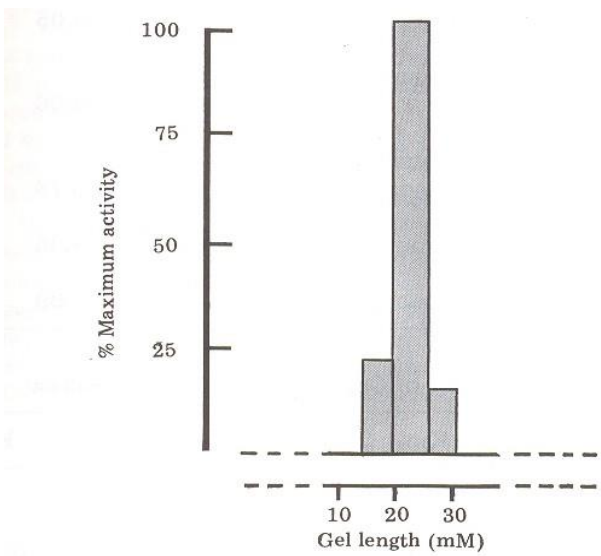

Fig. 2 Distribution of purified α-L-fucosidase activity in acrylamide gel.

Table – 1 Purification steps of alpha-L-Fucosidase

Purification	Enzyme Activity (nmol.min)		Protein (mg)		Specific Activity nmol/min/ mg	Yield (%)	Purification
	In 50 ul	Total	In 50 ul	Total			
Crude Homogenate	0.048	40.36	0.681	571.2	0.071	100	
$NH_4)S0_4$ ppt 30%	0.058	11.60	0.082	16.0	0.705	29	
$(NH_4)SO_4$ ppt 30-65%	0.181	27.13	0.361	54.0	0.503	67	
G-25 Column	0.024	23.10	0.065	54.0	0.370	57	
After elution with L fucose and Dialysis	0.030	12.40	0.001	0.26	42.860	30	604

Table 2. —Activity of five gylcosidases in purified a-L-Fucosidase

Gylcosidase	Substrate	% of a-L-Fucosidase Activity
β-D-Glucosidase 4.0	4MU- β -D-giucopyranoside	0.04
β -D-Glucosidase 5.3	4MU- β -D-glueopyranoside	0.02
α-L-Arabinosidase	4MU-α-L-arabinoside	0.05
β -D-Glucuronidase	4MU- β -D-glueuronidetrihydrate	0.06
N-Acetyl-β-D-glucosaminidase	4MU-2-acetamido-2-deoxy- β -D-glycopyranoside	0.78
α-D-Mannosidase 4.4	4MU- α -D-mannopyranoside	0.06
α-D-Mannosidase 6.4	4MU- α -D-monnopyranoside	0.09

Table 3. — Kinetic parameters of Crude and Purified Fucosidase

	Km	Vmax	Ki
Crude Fraction	0.045 ± 0.004	1.255±0.014	
Purified Fraction	0.052 ± 0.004	25.130 ± 0.063	0.46

Electrophoresis and Purity of Enzyme

The essential homogeneity of the purified enzyme was indicated by polyacrylamide rod gel electrophoresis under the conditions described in the 'methods'. The crude and purified enzyme exhibited the same electrophoretic mobility. The purified enzyme exhibited protein and enzyme activity bands at an identical position. The enzyme activity located in the gel is shown in Fig-2. With reference to various protein markers SDS electrophoresis indicated the presence of a single band of molecular weight of 50,000. A similar observation was reported from human liver[6] and rat liver[2] α-L- fucosidase.

Discussion

α-L-Fucosidase has been purified from several mammalian tissues (2, 6, 7, 8) but it has not been previously purified from skeletal muscle. From the present study it is apparent that a-L-fucosidase can be purified from skeletal muscle by using an affinity column (agarose-ACFA) as has been used to purify human liver fucosidase.[6] The present findings show that α-L-fucosidase from rabbit gastrocnemius muscle was purified to homogeneity. The purified α-L-fucosidase was essentially free of other glycosidases and has a pH optimum between 6.1-6-4 and exhibiting 50% of its maximal activity at pH 5.4 and 7.2. The purified α-L-fucosidase gave a single protein band on SDS-gel electrophoresis with a molecular weight corresponding to 50,000. The enzyme has been reported to exists in a number of sub-units. Rat epididymis enzyme has been reported to consist of two types of subunits,[8] whereas human brain and liver fucosidases have been reported to have a single type of sub-unit.[6,7] In the present investigation thermostability, pH activity profile, and kinetic studies also indicate the presence of a monomeric form of enzyme in rabbit skeletal muscle. It is further substantiated by disc gel electrohoresis that it contained one major protein component coinciding with the location of the enzyme activity. The substrate specificity of the multiple forms of fucosidase has not been studied extensively therefore, certain difference observed could have been resulted from the various sources of the enzyme preparations. The study of amino acids composition and carbohydrate contents of rat skeletal muscle fucosidase is anticipated and hopefully would shed light on the molecular nature of this enzyme. The improved method of purification of α-L-fucosidase described here would be of great significance in providing larger yield of homogeneous enzyme toward possible enzyme-replacement therapy in fucosidosis.

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