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HUMAN α-FUCOSIDASE

PURIFICATION AND PROPERTIES

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Summary

Human placental α-fucosidase (EC 3.2.1.51) has been extensively purified and partially characterized with respect to kinetic and structural properties. Although the enzyme seems to be separated by DEAE-cellulose chromatography in two forms which differ in their molecular weight and thermostability, an interconversion between the two forms takes place during storage and/or electrofocusing so that the same peaks of activity, revealed by the latter technique, are found before and after DEAE-cellulose chromatography, and also in the two forms separated on DEAE-cellulose. The heterogeneous peaks of activity revealed by isoelectrofocusing show a reproducible pattern in the different tissues examined, except in serum where their pI values are consistently more acidic.

Introduction

α-Fucosidase (EC 3.2.1.51) activity has been identified in different animal tissues [1,3] and two forms of this enzyme, characterized by different molecular weights, have been demonstrated in pig kidney [4]. Two similar forms of α-fucosidase have been shown in human liver, kidney, placenta and amniotic fluid [5,6]. The first one (I) is a macromolecular form excluded from Sephadex G-200 and it probably represents an aggregate of the second form (II), with an associated change in surface charge [6]. A deficiency of α-fucosidase activity is present in patients affected with fucosidosis [7], an inborn error of metabolism first observed in families of South-Italian origin [8]. Among the fucose-containing glycolipids and glycoproteins accumulated by these patients [7,9,10], a sphingolipid containing fucose, galactose, glucose, *N*-acetyl-glucosamine and ceramide in the ratio 1 : 2 : 1 : 1 : 1 has been reported [9]. This composition is common to both H and Lewis blood group substances [11], and in some patients, a strong Le^a activity has been noticed in red cells and sera [12].

Recently a partial purification by affinity-chromatography of α-fucosidase from human placenta and liver has been described [13,14]. Using this and

other classical steps, we purified the α-fucosidase from human placenta and separated it in different forms by isoelectrofocusing. These forms are all similar with respect to their K_m values and differ only for their charge properties as shown also by Cellogel electrophoresis and disc-gel electrophoresis. The different forms of placental α-fucosidase purified and characterized in the present study have also been identified in human liver, fibroblasts, sera and lymphoids cells [15—17].

Materials and Methods

The α-fucosidase activity was assayed using two different substrates: (a) *p*-nitrophenyl-α-L-fucoside (Nph-Fuc), 1 mM, in a final reaction mixture of 0.15 ml containing 15 μmol of citric acid/citrate buffer pH 5.5 and up to 50 μl of enzyme, incubated at 37°C for 30 min. The reaction was terminated by adding 0.85 ml of 0.2 M K_2CO_3 and the absorbance at 400 nm was read in a Beckman DU spectrophotometer; (b) 4-methyl-umbelliferyl-α-L-fucoside (Me-Umb-Fuc), 50 nmol in a final reaction mixture of 80 μl containing 7.5 μmol of citric acid/citrate buffer pH 6.0 and up to 30 μl of enzyme incubated at 37°C for 30 min. The reaction was terminated by the addition of 4 ml of 0.2 M K_2CO_3 and the fluorescence was measured in a Turner fluorimeter Model 101 with excitation at 360 nm and emission peak at 450 nm. Under the conditions used, the assay was linear either with time or with increasing enzyme concentration up to a limit represented by 1.25 μmol of reaction product. Thermal inactivation of the different forms of the enzyme was performed at 51°C and pH 6.5 in potassium phosphate buffer 0.05 M. One milliunit of enzymatic activity corresponds to 1 nmol of the indicated substrate cleaved in 1 min at 37°C. Specific activity is indicated as milliunit per mg of protein.

Isoelectrofocusing was performed with a 110 ml column (LKB electrofocusing equipment). The carrier ampholyte concentration was 0.8% with a pH range from 5 to 7 for all experiments and 4 to 6 when serum was run. The temperature was maintained at 4°C. Voltage was started at 600 V and increased stepwise to 1000 V over 6 h. The run was completed in about 23 h and the column was slowly drained and collected in 1.5-ml aliquots. The pH was measured with a Radiometer pH-meter 26 at room temperature. Electrophoresis was carried out on Cellogel strips (500 μ 16 × 16 cm, Chemetron, Italy) in 40 mM Veronal buffer pH 8.6 at 25 mA for 150 min. After the run the strip was blotted and soaked in 0.3 mM fluorogenic substrate (dissolved in 0.1 M citric acid/citrate buffer at pH 5.5) and incubated in a moist chamber for 1 h at 37°C. After disc-gel electrophoresis [18] duplicate gels were similarly stained for α-fucosidase activity and for protein.

Separation of the thermostable and thermolabile forms was obtained by chromatography on a column of Sephadex G 200 (1.5 × 100 cm) with a flow rate of 3 ml/h. The eluting buffer was phosphate 50 mM, pH 6.0.

The determination of molecular weights by thin-layer gel filtration was performed on 0.6 mm layers of Sephadex G-200 Superfine, prepared as described [19]. Neuraminidase treatment of placental α-fucosidase was done as already reported for α-galactosidase [20].

Agarose-epsilon-amino-caproyl-fucosamine used for the affinity column (see

Results) was purchased from Miles-Yeda Ltd.; Nph-Fuc and Me-Umb-Fuc from Koch-Light.

Results

Purification of α-fucosidase

A typical scheme of purification of α-fucosidase is reported in Table I. Placentas from normal deliveries which were kept frozen at −20°C until used, were washed in H_2O, freed from umbilical cords and membranes, then diced and homogenized in a Waring-Blender using 2 vols. of 0.05 M potassium phosphate buffer pH 6.5. The homogenate was centrifuged at 10 000 × *g* for 30 min and the successive steps (ammonium sulphate fractionation, ethanol precipitation, zinc sulphate precipitation and DEAE-cellulose chromatography) were performed as already described for the purification of human α-galactosidase [20]. The DEAE-cellulose chromatography was developed using a gradient of NaCl concentration (from 0.04 M to 0.1 M for 24 h and from 0.1 M to 0.4 M for other 24 h) in potassium phosphate buffer 0.01 M pH 6.5. About one-third of α-fucosidase activity was not absorbed on the DEAE-cellulose column and was eluted before the starting of the gradient (peak A, Table I). The two peaks from DEAE-cellulose were precipitated by adding solid ammonium sulphate to a concentration of 60% and the pellet obtained by centrifuging at 6000 × *g* was resuspended and dialyzed against 50 mM potassium phosphate buffer pH 6.5. The affinity column (2 × 6 cm) was prepared in this buffer following a published procedure [14]. The purified preparation from this last step showed on disc-gel electrophoresis only a large band having α-fucosidase activity (Fig. 1). However, when different forms of α-fucosidase were separated by isoelectrofocusing, this large band was resolved into bands with slightly different mobility on disc-gel (see below).

The activity of α-fucosidase preparations purified by affinity chromatography was doubled when an aliqout of 10 μg of bovine serum albumin was added to the standard assay.

TABLE I

PURIFICATION OF α-FUCOSIDASE

	Vol (ml)	Protein (mg/ml)	Nph-Fuc activity (mU/ml)	Total activity (mU)	Specific activity (mU/mg)	Purif. (-fold)	Yield (%)
Crude extract (Sup. 10 000 × *g*)	10 000	33	96	960 550	2.9	—	—
After $(NH_4)_2SO_4$	1 000	83	720	720 400	8.6	3.0	75
After ethanol precipitation	1 290	25	398	514 000	16.0	5.5	53.5
After $ZnSO_4$ precipitation	1 340	21	318	426 566	16.6	6.1	44.4
After DEAE-Cell chromatography							
peak B	12.3	57	12 080	148 625	212	73.1	15.5
peak A	50	92	1 479	73 950	16	5.5	7.7
After affinity chromatography							
peak B	73.8	0.22	700	51 660	3 181	1 100	5.4
peak A	15	0.88	1 041	15 615	1 183	408	1.6

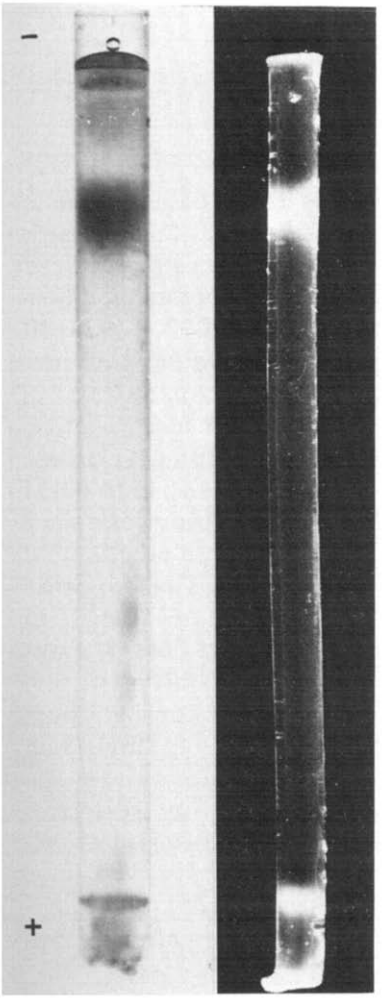


Fig. 1. Disc-gel electrophoresis of whole α-fucosidase (form B not separated by isoelectrofocusing) after the affinity chromatography step. Left: staining for proteins and right for enzymatic activity. Gels of 15 cm of length were used for this experiment. A preparation of purified A form gave an identical band.

Characterization of different α-fucosidase forms

The two peaks of α-fucosidase separated on DEAE-cellulose and further purified by affinity chromatography could be easily distinguished by their different rates of heat inactivation at 51°C, as shown in Fig. 2. The protein content of the purified preparation used for this experiment was equal to 0.88 mg/ml and 0.63 mg/ml respectively for the unadsorbed and adsorbed peak on DEAE-cellulose. Following a nomenclature commonly used for the multiple forms of lysosomal enzymes, the thermolabile peak (unadsorbed) and the thermostable one (adsorbed) have been labeled respectively A and B. The addition of bovine serum albumin was shown to stabilize the thermolabile A form while the thermostable B form remained almost unchanged (Fig. 2). On the other hand, in presence of a chaotropic agent such as potassium thyocyanate [21] (final con-

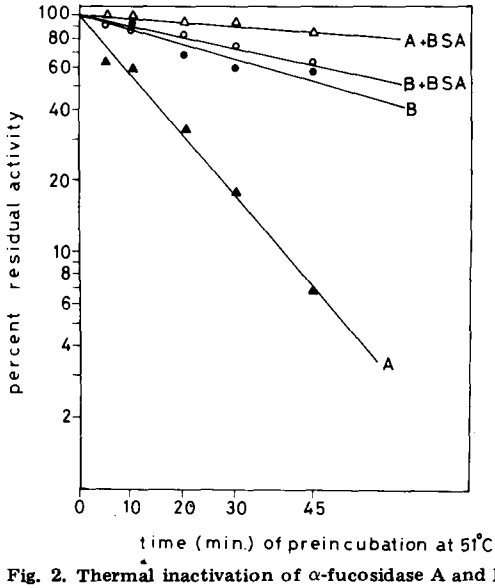


Fig. 2. Thermal inactivation of α-fucosidase A and B purified through affinity chromatography. The purified preparation of α-fucosidase A and B had a protein content of 0.88 mg/ml and 0.63 mg/ml respectively. Bovine serum albumin (BSA) was added to an aliquot of these preparations so that their protein content reached approx. 2.5 mg/ml.

centration 0.5 M) both forms were thermolabile. Mixtures of the two forms, without any addition of stabilizing or dissociating agents, yielded double rates of heat inactivation which reflected the relative amounts of the two forms. Storage for three months at 4°C increased the relative proportion of the thermostable form, as revealed by heat inactivation and by gel filtration.

A typical isoelectrofocusing pattern of the partially purified enzyme from the second peak of the DEAE-cellulose chromatography (B form) is shown in Fig. 3. The activity and the relative proportions of the different forms were not significantly different when assayed with the two substrates Me-Umb-Fuc and Nph-Fuc. The exception represented by peaks 3 and 4 (Fig. 3) is only apparent because, as already mentioned under Methods, the fluorogenic assay is no longer linear beyond 1.25 nmol of reaction product (equal to 15 000 Fluorescent Units). This pattern was completely identical to that present in the enzyme preparation before the DEAE-cellulose step. In one experiment in which Ampholines pH 4—6 were used, an additional small peak having a p*I* of 4.9 was found. However, the four major forms with p*I* values corresponding to 5.39, 5.63, 5.94 and 6.24 respectively were constantly present. These values are the averages obtained from four different experiments performed at various stages of purification (Table II). In addition, the K_m values of the different forms were not significantly different for the two substrates used (Table II).

The pH optima of enzymatic activity, which were 5.5 for the Nph-Fuc substrate and 6.0 for the Me-Umb-Fuc substrate, were the same for the five forms. The relative electrophoretic mobility of the different forms on disc-gel and Cellogel is shown in Fig. 4. The migration on disc-gel is in agreement with the pattern expected from isoelectrofocusing, the form with the highest p*I* (N° 5) being the slowest at the pH used for this electrophoresis. However on Cellogel

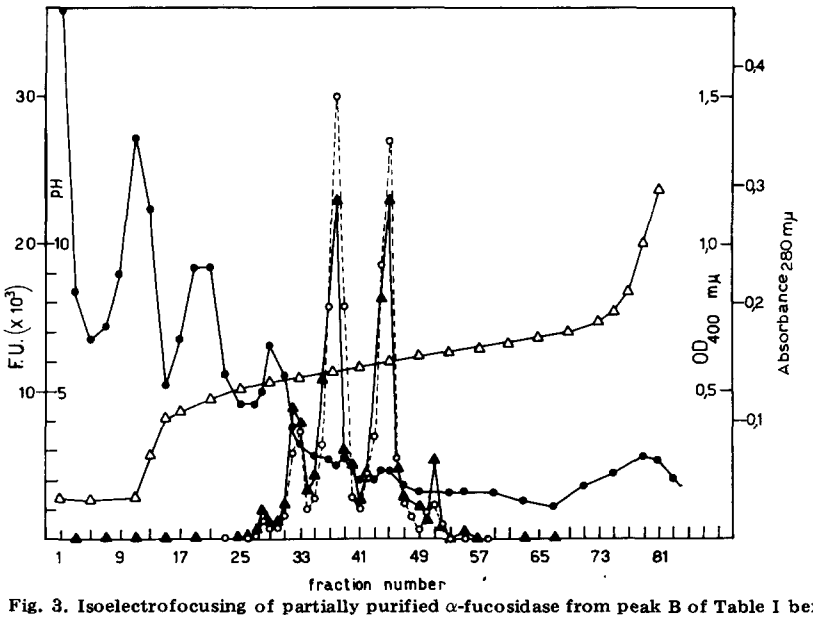


Fig. 3. Isoelectrofocusing of partially purified α-fucosidase from peak B of Table I before affinity chromatography. △——△, pH; ●——●, absorbance 280 nm; ○——○: absorbance 400 nm; ▲——▲: fluorescent units.

electrophoresis this pattern is completely reversed. The possible interaction between the enzymatic protein and the Cellogel is discussed below as a cause of this modified migration. The four major forms purified from placenta (N° 2, 3, 4 and 5) were further characterized by thin-layer gel filtration (see Methods) to determine their molecular weight. The plot on semilog paper of the four markers used was linear and, after staining for α-fucosidase activity, all the major forms showed an enzymatic protein with molecular weight of 50 000. The three major forms with lower p*I* values (N° 2, 3, 4) presented also a fainter band with a molecular weight corresponding to approximately 200 000. This band could represent an aggregate of the former. The same kind of molecular

TABLE II

MULTIPLE FORMS OF α-FUCOSIDASE SEPARATED BY ISOELECTROFOCUSING

The p*I* values reported are the means of 4 different experiments. An additional minor peak with p*I* of 4.9 was found in one experiment under slightly different experimental conditions as described in the text. The apparent K_m values for the two substrates were calculated by the Lineweaver and Burk method.

Enzyme forms	p*I* values (± S.D.)	K_m for Nph-Fuc ($\times 10^{-3}$ M)	K_m for Me-Umb-Fuc ($\times 10^{-3}$ M)
Whole α-fucosidase	—	0.38	0.3
Form 1	5.19 ± 0.07	0.25	0.25
2	5.39 ± 0.04	0.29	0.38
3	5.63 ± 0.06	0.45	0.35
4	5.94 ± 0.07	0.73	0.65
5	6.24 ± 0.07	0.6	0.33

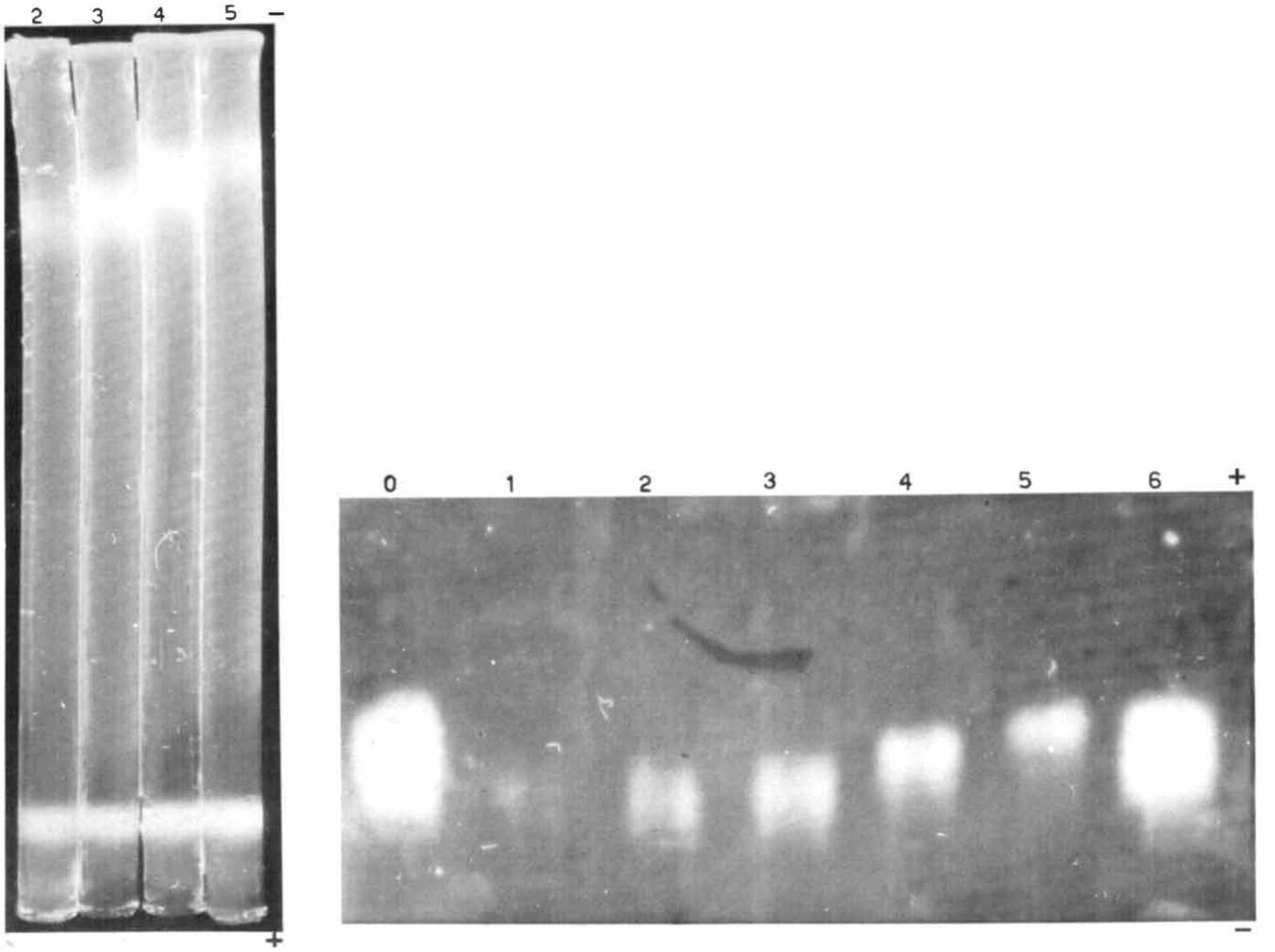


Fig. 4. Right: cellogel electrophoresis of the different forms of α-fucosidase activity from the isoelectrofocusing of Fig. 3. No. 0 and 6: α-fucosidase before isoelectrofocusing; 1, 2, 3, 4, 5: different forms separated by isoelectrofocusing. Left: disc-gel electrophoresis of forms 2, 3, 4, 5 from the same material. The gels used in this experiment were smaller (10 cm) than those used for the experiment reported in Fig. 1. Staining for α-fucosidase activity was obtained as described in the text. In this figure and in Fig. 1 the fluorescent band at the bottom of the gel is given by the bromophenol blue marker.

weight determination performed on the four major forms separated from serum of a normal control showed a similar composition consisting of two bands, one corresponding to a molecular weight of 127 000 and the other to a molecular weight of 64 000.

Isoelectrofocusing pattern in tissues and sera

Different human tissues analyzed by isoelectrofocusing (Fig. 5, A, B and C) showed a pattern of α-fucosidase forms quite similar to that found in placenta with respect to their pI values and to their relative activity (Table III). However, in serum, all the peaks from normal controls showed p*I* values which were more acidic than those found in other tissues (Fig. 5, D). Neuraminidase treatment of placental α-fucosidase caused only disappearance of the minor acidic peaks, thus confirming previous reports [15—17]. However, treatment with

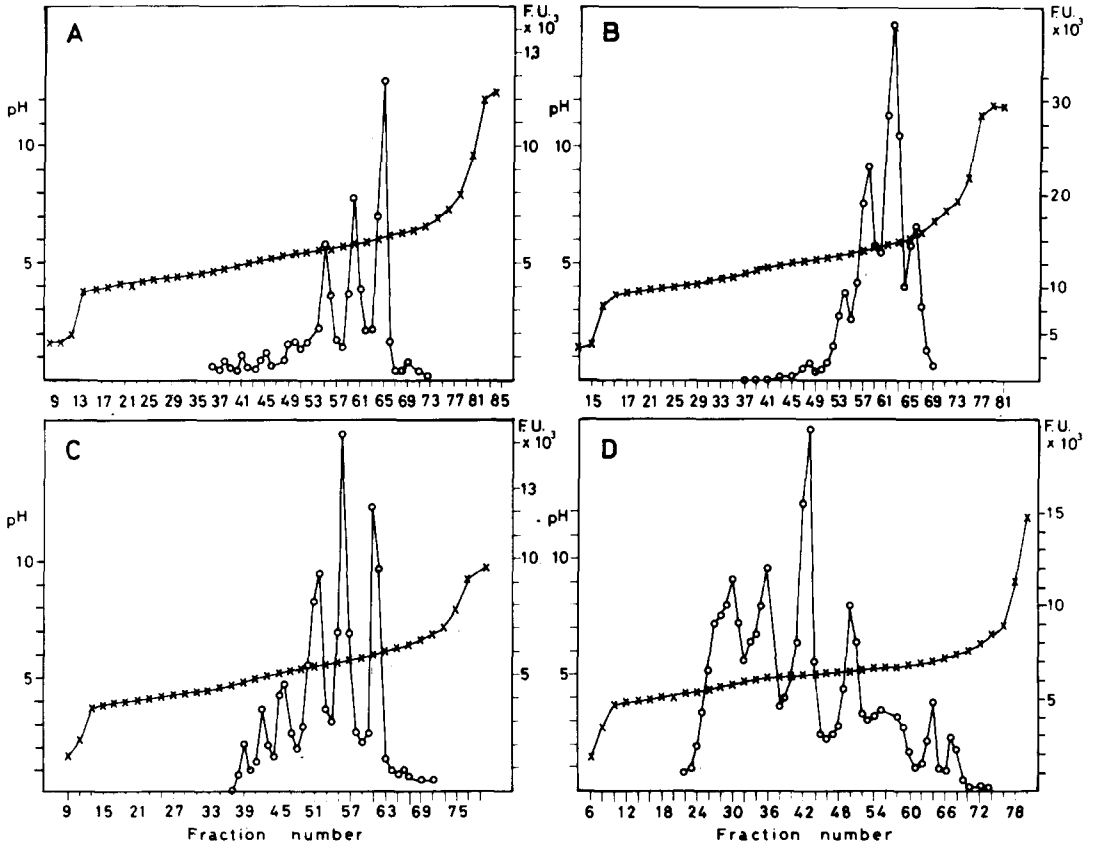


Fig. 5. Heterogeneity of α-fucosidase in different human tissues as shown by isoelectrofocusing. A, kidney; B, brain; C, heart; D, serum; F.U., fluorescent units of the α-fucosidase assay, as described under Methods. The enzyme from different tissues used in A, B and C was partially purified through the $(NH_4)_2SO_4$ and ethanol steps (see Table I). In each of these experiments the column was loaded with a preparation containing 50—100 mU of activity and 150 mg of protein. The fluorogenic assay (reported on the abscissa) was run for 50 min with aliquots of 30 μl of enzyme, as described under Methods. In experiment D (serum) the column was loaded with 3 ml of serum containing 18 mU of activity and 200 mg of protein. The assay in this experiment was run under the same conditions as used above, for 7 h.

TABLE III

COMPARISON OF pI VALUES OF α-FUCOSIDASE ACTIVITY FROM DIFFERENT HUMAN TISSUES AND RELATIVE PROPORTION (%) OF EACH PEAK OF ACTIVITY IDENTIFIED BY ISOELECTROFOCUSING

Placenta		Kidney		Brain		Heart		Serum	
pI	%	pI	%	pI	%	pI	%	pI	%
—	—	—	—	—	—	—	—	4.56	27.8
—	—	4.7	2	—	—	4.77	2.6	4.8	24.4
—	—	4.9	2.2	—	—	4.96	5.5	5.01	24.4
5.19	6.5	5.12	3.8	5.17	1.4	5.17	8.9	5.15	11.4
5.39	16.2	5.38	7	5.4	9.2	5.45	25.2	5.3	5.9
5.63	32	5.6	20.5	5.62	29.9	5.64	36.7	5.58	3.7
5.94	33.5	5.8	27.5	5.87	41.6	5.9	21	5.8	2.4
6.24	8.7	6.1	36.9	6.2	17.9	—	—	—	—

neuraminidase of sera from normal controls caused a shift of all the peaks towards less acidic pI values, as described in the accompanying paper [22].

Discussion

The separation of human α-fucosidase in two peaks by ion exchange chromatography has been interpreted as due to different states of aggregation of the two forms identifiable by gel filtration with an associated change in surface charge [6]. The information added now by this report and by another [17] shows that the two different types of molecular aggregates separated by gel filtration or by DEAE-cellulose chromatography yielded by isoelectrofocusing four similar major peaks of α-fucosidase activity and some minor ones. Moreover, the pattern and the relative proportions of the different peaks found by isoelectrofocusing are the same before and after DEAE-cellulose chromatography. In addition, gel filtration yields two forms of α-fucosidase activity [5], the first one (I) characterized by a molecular weight of 200 000 and by its thermostability, the second (II) by a molecular weight of 50 000 and its thermolability (See Table IV). In presence of bovine serum albumin this latter thermolabile form becomes thermostable, while in presence of a dissociating agent such as potassium thyocyanate both forms are thermolabile. It has already been reported that form II, which is thermolabile when highly purified, is thermostable in preparations with a relatively high protein concentration [5]. This effect has been reproduced by the addition of bovine serum albumin.

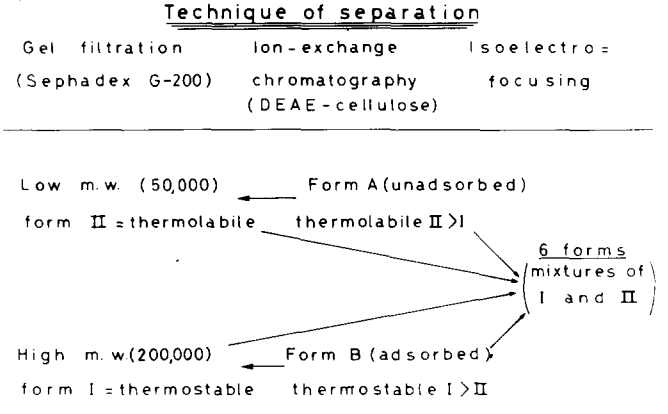
The experiments presented in this paper and reported in the literature [5,6, 17] suggest an interconversion between the thermostable high molecular weight form and the thermolabile low molecular weight form. The former is probably an aggregate of the latter, as the experiments performed with thiocyanate indicate. Since no difference can be found in the patterns of peaks revealed by isoelectrofocusing before and after DEAE-cellulose, it is necessary to postulate an equilibrium between the two forms which is reestablished during isoelectrofocusing (See Table IV).

The activity of purified preparations of α-fucosidase is influenced by its protein content, as shown by the doubling of activity upon addition of bovine

TABLE IV

POSSIBLE INTERRELATIONSHIP BETWEEN THE DIFFERENT FORMS OF α-FUCOSIDASE BASED ON TWO DIFFERENT STATES OF AGGREGATION, AS DEDUCED FROM GEL FILTRATION.

An equilibrium between the two different states of aggregation should be postulated to justify the failure to isolate the low (I) and high (II) molecular weight aggregates by isoelectrofocusing. These are partially separated by DEAE-cellulose chromatography, on the basis of their charge differences, but each form results were contaminated by the other, as indicated by their proportions (II > I or I > II).



serum albumin. This effect has been demonstrated for other glycosidases as well [20]. Eventually, the discrepancy noticed between the migration on Cellogel and on disc-gel electrophoresis has been reported also for the two forms of α-galactosidase [20]. The phenomenon is probably related to the glycoprotein nature of these enzymes, and to their interaction with the supporting media used for electrophoresis.

The previously unnoticed finding of heterogeneity between serum and other tissues is probably due to different numbers of sialic acid residues, as discussed in the accompanying paper, [22].

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