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SYSTEMATIC USE OF AFFINITY DIFFERENCES BETWEEN IMMOBILIZED LECTIN GELS FOR DEMONSTRATION OF GLYCOPROTEIN MOLECULAR VARIANTS

THE EXAMPLE OF RADISH β-FRUCTOSIDASE

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SUMMARY

The glycoprotein β-fructosidase from radish seedlings was used as an example. The extended charge heterogeneity of this enzyme does not allow analytical investigations on the microheterogeneity of the carbohydrate moiety by lectin crossed affinity immunoelectrophoresis. This microheterogeneity was investigated by a new approach of affinity chromatography on to columns of immobilized concanavalin A and *Lens culinaris* agglutinin displaying different affinities. This series of gels allowed a new approach for the detection and control of the microheterogeneity of the glycoprotein sugar moiety. The general chromatographic procedure requires (1) flat gradient elution to observe the heterogeneous forms, (2) re-chromatography and/or crossed chromatography for monitoring the biological significance of the heterogeneous elutions observed and (3) systematic "crossed chromatography" for the detection of further heterogeneous forms.

INTRODUCTION

Molecular heterogeneity in the carbohydrate moiety of glycoproteins has often been investigated by lectin affinity experiments. Crossed affinity immunoelectrophoresis (CAIE) with lectin has been shown to be a powerful technique[1–3] and a recent improvement, carbohydrate electroendosmotic elution, increased its sensitivity[4]. However, this CAIE technique cannot always be used without purified preparations as a control[4] and also it requires either monospecific antisera or biological charac-

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terization for molecular variant detection. Another limitation to CAIE analysis is charge heterogeneity: commonly observed with particular glycoproteins and even suspected for some lectins in CAIE conditions, this phenomenon can produce heterogeneous immunoprecipitation patterns without any relation to lectin binding heterogeneity. Hence chromatography on immobilized lectin columns may, in some instances, be the only method for the study of carbohydrate variants. The wellknown drawbacks of this technique, as generally observed for affinity chromatography, are primarily non-biospecific interactions with the ligand or gel matrix (for a review, see ref. 5), but precautions to avoid non-sugar-specific binding on immobilized lectin columns have also been described[6,7].

In this paper, a new application of column affinity chromatography is described for the characterization and isolation of glycoprotein molecular variants; it takes advantage of (1) affinity differences between two immobilized concanavalin A (Con A) gels, (2) crossed chromatography and (3) the flat gradient elution technique. Extensive studies of sugar-specific interactions have shown that elution profiles obtained from high- or low-affinity columns are not linked to enzyme charge or mass heterogeneity or to carbohydrate degradation, so that good evidence is provided for the microheterogeneity of the glycoprotein sugar moiety.

In our hands, radish β-fructosidase (β-FFase) has proved to give a good example of the technique: this enzyme is a glycoprotein[8] containing 7.7% of carbohydrates[9] and displaying largely heterogeneous behaviour on agarose gel electrophoresis[10], which hindered its study using lectin CAIE. The method reported here allowed us to observe and isolate three carbohydrate variants of β-FFase.

EXPERIMENTAL

Chemicals

The two different immobilized lectins used in this study, Con A and *Lens culinaris* agglutinin (LCA), were purchased either from Pharmacia (Con A-Sepharose, batch 18812; LCA-Sepharose, batch 7767) or from Reactifs IBF, Pharmindustrie (Con A-Ultrogel, batch L.593; LCA-Ultrogel, batch L.127). Methyl α-mannoside (Me-α-Man) was obtained from IBF and all other chemicals from Merck.

Enzyme source and assay

β-FFase was isolated from light-grown radish seedlings irradiated for 72 h with a standard far-red light source[11]. The crude enzyme preparation used here was obtained after Sephadex G-25 (Pharmacia) chromatography of radish seedling extract as described elsewhere[9]. β-FFase activity was measured as described elsewhere[9].

Affinity chromatography on to immobilized lectin columns

All affinity chromatography was performed at room temperature. The lectin columns (22 × 1.6 cm I.D.) were equilibrated with 85 mM citrate phosphate buffer (pH 6.5) containing 1 *M* sodium chloride, 1 m*M* magnesium chloride, 1 m*M* manganese chloride and 1 m*M* calcium chloride. Enzyme samples (0.5 ml) were made from freeze-dried powder dissolved in the same equilibration buffer and were applied on to the columns after centrifugation (3100 *g*, 15 min). After washing the gel with the same buffer, the bound glycoproteins were first eluted with a linear Me-α-Man

gradient (0 to 2.5 m*M*, total volume 100 ml). After an equilibration step with at least 25 ml of 2.5 m*M* Me-α-Man, a second 100-ml gradient from 2.5 to 100 m*M* Me-α-Man was sometimes applied. For the standard procedures presented here, a stepwise 100 m*M* Me-α-Man elution was prefered. The columns were eluted at 46 ml/h, the eluate was monitored with an ultraviolet densitometer at 280 nm, collected in 3.6-ml fractions and measured for β-FFase activity. Prior to re-chromatography, if any, the fractions in a given peak of β-FFase activity were pooled, concentrated on an Amicon PM 30 ultrafiltration membrane and finally dialysed against the equilibration buffer. The retardation coefficient (R_c) is expressed here as V_e/V_i, where V_e is the elution volume of β-FFase activity on lectin columns in the presence of equilibration buffer and V_i is the enzyme elution volume when β-FFase was chromatographed on the same column in the presence of 100 m*M* Me-α-Man.

RESULTS

Elution profiles on Con A columns

When chromatographed on Con A-Sepharose, β-FFase was first completely retained on this column and then partly (90%* of the recovered enzyme activity) eluted as a sharp peak (I_s) in the first volumes of a gentle linear Me-α-Man gradient (0–2.5 m*M*) as shown in Fig. 1. A second enzyme form (II_s = 10%) was eluted homogeneously with approximately 25 m*M* of this competing glycoside, as calculated from a second linear gradient (2.5–100 m*M* me-α-Man) (not shown). Finally, more than 90% of the enzyme activity in the originally applied material was recovered; the residual activity could not be eluted from the column with higher Me-α-Man concentrations.

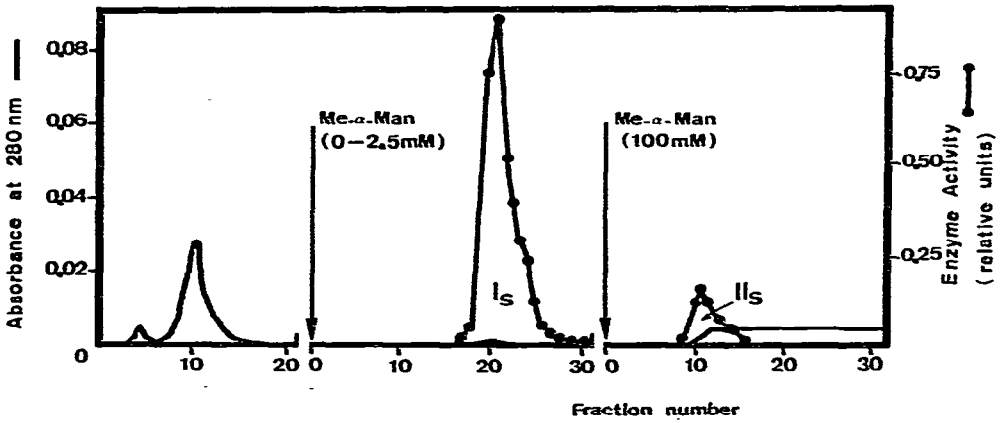

Fig. 1. Con A-Sepharose elution profile of β-FFase (crude enzyme preparation). Fraction size, 3.6 ml. Starting buffer, 85 m*M* citrate phosphate buffer (pH 6.5) containing 1 *M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$. First arrow indicates start of elution with linear Me-α-Man gradient (50 ml × 2 from 0 to 2.5 m*M*). Second arrow indicates start of elution with buffer containing 100 m*M* Me-α-Man. The first enzyme peak (I_s) represents 90% and the second peak (II_s) 10% of the recovered enzyme activity.

* All values are the means of at least three experiments performed on the same column.

This result was strengthened by Con A-Ultrogel chromatography (Fig. 2A). β-FFase elution profiles on this lower affinity column[12] allowed one to distinguish (a) an unbound form (I_u) representing 57% of the recovered enzyme activity and eluted as a highly retarded trailing peak with equilibration buffer lacking Me-α-Man; the heterogeneity in the elution profile of this first enzyme form was highly reproducible and reflected different affinity levels (R_e 1.4, 1.7, 2.0); (b) a loosely bound form (II_u; 43%) which was eluted in the first volumes of the Me-α-Man gradient (0–2.5 m*M*) as a sharp peak (Fig. 2A). No more activity was eluted with 100 m*M* of this competiting glycoside.

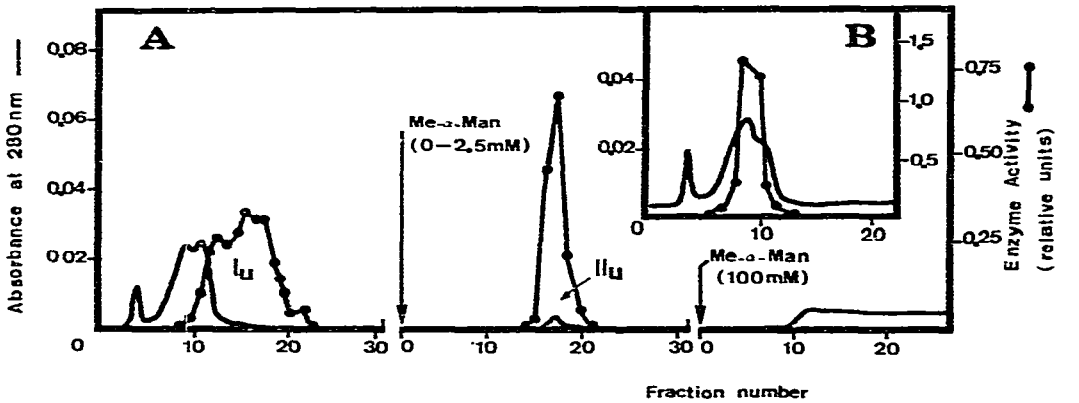

Fig. 2. Con A-Ultrogel elution profile of β-FFase (crude enzyme preparation). (A) Fraction size and elution conditions as in Fig. 1. The first peak (I_u showing retarded elution) represents 57% and the second peak (II_u) 43% of the recovered enzyme activity. Starting buffer, sample and column contained 100 m*M* Me-α-Man. The whole enzyme activity was unbound and eluted without any retardation when compared with elution of other extract proteins.

Establishment of biological significance of heterogeneous elution profiles

The two fractions isolated from either Con A column were eluted in their original position when re-chromatographed separately on the same column. Further, the whole of the enzyme activity was recovered with the unretained proteins when the sample, column and equilibration buffer were supplemented with 100 m*M* Me-α-Man, whatever the gel in use; in particular, the heterogeneity and retardation of the first peak on Con A-Ultrogel completely disappeared under the last experimental conditions (see Fig. 2B, insert). Hence the heterogeneity observed in elution profiles on Con A columns did not result from (1) column overloading, (2) non-sugar-specific interactions with the immobilized lectin or (3) sieving effects.

Elsewhere, the Con A-Ultrogel-retarded fraction (I_u) was completely bound on Con A-Sepharose and subsequently eluted as a sharp peak with a 0–2.5 m*M* Me-α-Man gradient, indicating that degradation in the β-FFase carbohydrate moiety occurring before or during Con A-Ultrogel chromatography could not account for the weak affinity observed on this last gel.

Finally, all chromatographic runs were performed in the presence of a high sodium chloride concentration (1 *M*) to prevent enzyme electrostatic interactions with Con A or the gel matrix, as the latter case has already been described for β-FFase from different origins[9,13].

When β-FFase was chromatographed on immobilized LCA, it was unretained on LCA-Ultrogel and not adsorbed but specifically retarded on the LCA-Sepharose column (R_c 1.13) (not shown). The very different elution profiles obtained from Con A and LCA immobilized on the same matrix give new evidence to exclude non-specific interactions with the matrix and/or sieving effects as an explanation of heterogeneous profiles.

Crossed chromatography for detection of microheterogeneity

The different ratios observed for low- and high-affinity forms on each column (see Table I) were indicative of greater complexity than was inferred from a single elution profile exhibiting two molecular variants. To obtain more precisely the relationships between the molecular variants isolated from each column chromatography, the first and second forms obtained from Con A-Sepharose (I_s and II_s, respectively) were re-chromatographed separately on Con A-Ultrogel. The fraction showing the highest affinity for Con A-Sepharose (II_s) was completely retained on Con A-Ultrogel and was eluted as a single peak with Me-α-Man; however, as shown in Fig. 3, a β-FFase form of lower affinity on Con A-Sepharose (I_s) could be further divided

TABLE I

SUMMARY OF THE VARIOUS FRACTIONS ISOLATED FROM CON A-SEPHAROSE OR CON A-ULTROGEL CHROMATOGRAPHY, RE-CHROMATOGRAPHY AND CROSSED-CHROMATOGRAPHY

Results expressed as percentage of total enzyme activity recovered from the column.

Sample	*Immobilized lectin*	*Fraction*			
		Unretained, unretarded	*Unretained but retarded*	*Retained and eluted with 0–2.5 mM Me-α-Man*	*Retained and eluted with 100 mM Me-α-Man*
		Single chromatography:			
CEP*	Con A-Sepharose	—	—	(I_s) 90	(II_s) 10
CEP+sugar**	Con A-Sepharose	100	—	—	—
CEP	Con A-Ultrogel	—	(I_u) 57 (R_c 1.4, 1.7, 2.0)	(II_u) 43	—
CEP+sugar	Con A-Ultrogel	100	—	—	—
CEP	LCA-Sepharose	—	100 (R_c 1.13)	—	—
CEP+sugar	LCA-Sepharose	100	—	—	—
CEP	LCA-Ultrogel	100	—	—	—
		Re-chromatography on the same column:			
$_s$***	Con A-Sepharose	—	—	100	—
I_s	Con A-Sepharose	—	—	—	100
$_u$	Con A-Ultrogel	—	100	—	—
I_u	Con A-Ultrogel	—	—	100	—
		Crossed chromatography:			
$_u$***	Con A-Sepharose	—	—	100	—
$_s$	Con A-Ultrogel	—	45	55	—
I_s	Con A-Ultrogel	—	—	100	—

* CEP = crude enzyme preparation.

** CEP+sugar: chromatography performed in 100 m*M* competing glycoside (Me-α-Man).

*** I_s, II_s, I_u, II_u: fractions used separately for re-chromatography or crossed chromatography.

into a non-adsorbed, retarded fraction (45%) and a bound fraction (55%) when re-chromatographed on to Con A-Ultrogel. Further studies of these last elution patterns showed them to be linked to sugar-specific interactions with the immobilized lectin. This result, obtained from crossed chromatography, is indicative of at least three molecular variants of β-FFase.

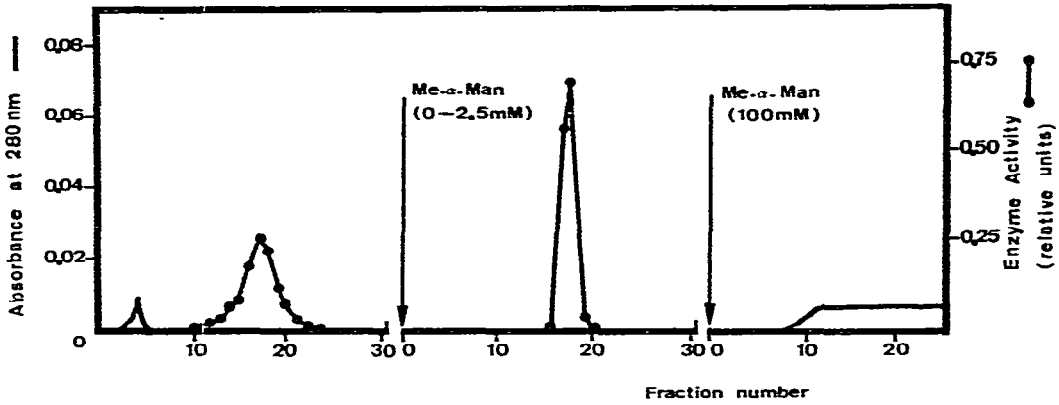

Fig. 3. Re-chromatography on to Con A-Ultrogel of β-FFase made of the first major peak (I_1) obtained from Con A-Sepharose chromatography (see Fig. 1). Fraction size and elution conditions as in Fig. 1. The first peak (showing retarded elution) represents 45% and the second peak 55% of the recovered enzyme activity.

DISCUSSION

Preliminary observations obtained from immunochemical analysis[1-4] and affinity precipitation techniques showed that β-FFase charge heterogeneity may arise from microheterogeneity in the sugar moiety of this enzyme; in contrast with the complete specific enzyme precipitation obtained when using glutaraldehyde cross-linked Con A as affinity adsorbent, only partial precipitation of β-FFase activity was observed when the largest amount of soluble Con A was used[8]. In order to obtain a better understanding of β-FFase microheterogeneity, we developed a new application of lectin affinity chromatography for the detection and isolation of molecular variants that differ only slightly in their affinity for lectins.

It is not our purpose here to investigate the origin of affinity differences between Ultrogel- and Sepharose-bound lectins. First reported by Kerckaert and Bayard[12] with Con A-reactive rat α-fetoprotein, in the present study these differences were confirmed for Con A, and also for LCA in the case of radish β-FFase interactions. Differences in the binding techniques used for lectin immobilization either on cyanogen bromide-activated Sepharose or on glutaraldehyde-activated Ultrogel probably induced changes in lectin affinity by slight conformational alterations of the interacting site. We report here that these changes in the affinity of immobilized Con A make available an immobilized Con A (Con A-Ultrogel) whose binding affinity is intermediate between those of Con A-Sepharose and LCA-Sepharose ones. Consequently, our chromatographic method is based on the use of this series of gels: systematic chromatography of the sample on to each of these gels, completed by crossed chromatography of given fractions from one gel on to another allowed us to

observe and isolate further microheterogeneous forms of β-FFase, not detectable from a single affinity chromatography.

Few results have been reported on the use of gradient elution on lectin columns[15–17]. Preliminary investigations with this technique allowed us to use the lowest concentrations of eluting glycoside to obtain the highest purification factor for β-FFase from Con A-Sepharose chromatographic steps[9]. Here, a flat gradient is shown to be extremely helpful in investigating affinity differences and in distinguishing among molecular variants of glycoproteins.

The literature provides many examples of non-specific adsorption to immobilized lectins[18]. In our study care was taken to avoid such artefacts; in particular, the use of 1 *M* sodium chloride in the buffer prevented ionic interactions. Further, extensive studies including re-chromatography and crossed chromatography allowed us to exclude enzyme degradation, binding capacity saturation, gel sieving effects and non-sugar-specific interactions as explanations for elution profile heterogeneity. Hence our experimental conditions provide good evidences for β-FFase molecular microheterogeneity in oligosaccharide side-chains, with at least three molecular variants of this enzyme. Further analysis of radish β-FFase lectin-binding heterogeneity is now in progress in relation to the structure, localization and charge variations of the enzyme.

In summary, we have developed a new approach for the detection and control of the microheterogeneity of the glycoprotein sugar moiety. This approach takes advantage of presently available immobilized lectin gels displaying different affinities for a given glycoprotein and requires (1) flat gradient elutions to observe the heterogeneous forms, (2) re-chromatography and/or crossed chromatography for study of the biological significance of the heterogeneous elutions observed and (3) systematic crossed chromatography for the detection of further heterogeneous forms.

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