Self-associating properties of yeast exo-β-glucanases are dependent on the ionic strength

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Glucanase (exo-β) *M_r-determination* *Self-association* *Ionic strength, of elution buffer* *Gel exclusion chromatography* *Yeast glucanase*

1. INTRODUCTION

1,3-β-D-Glucanases have been described in many yeasts of both ascomycetous and basidiomycetous origin [1–5]. The M_r values of these enzymes varies considerably from 10 000 to 200 000 but values between 20 000 and 60 000 are common for exo-β-glucanases. Yeast endo-β-glucanases tend to have a higher molecular weight [6].

It will be shown in this paper that the molecular weight determined by gel exclusion chromatography of exo-β-glucanases varies considerably depending on the ionic concentration of the elution buffer. The proteins undergo different degrees of self-association and are the highest when the columns are equilibrated with doubly distilled water. The aggregated enzymes are reversibly dissociated by the ionic strength of 0.025–0.1 M sodium acetate buffer but are not affected by 0.1% Triton X-100.

2. MATERIALS AND METHODS

Purified ferritin, thyroglobulin and ovalbumin were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and cytochrome *c*, α-glucosidase, β-glucosidase and α-mannosidase were purchased from Sigma Chemical Company (St. Louis, MO., USA). All other chemicals were of reagent grade.

2.1. *Organism and growth conditions*

Saccharomyces cerevisiae strains X_{14}, ts^{-136} *Me10* [7] and S288C (ATCC 26108) were used. *Candida utilis* (CECT 1061) and *Geotrichum lactis* (CECT 1102) were obtained from the Spanish Type Culture Collection, Salamanca, Spain. The type strain (UCD, FS and T 50-80) of *Kluyveromyces phaseolosporus* was obtained from the yeast culture collection of the Department of Food Science and Technology, University of California, Davis, USA. Microorganisms were grown as in [7].

2.2. *Enzyme preparations*

Exo-β-glucanases from *S. cerevisiae* strains X_{14} and ts^{-136} *Me10* were prepared as in [7], while those from *S. cerevisiae* strain S288C, *C. utilis*, *Kl. phaseolosporus* and *G. lactis* were obtained as reported in [8,9,2,10], respectively.

2.3. *Gel exclusion chromatography*

Gel exclusion chromatographies were performed in three columns packed, as recommended by the manufacturer, with Sephacryl S-200 (105.5 × 1.6 cm), Sepharose 4B (17.3 × 1.5 cm) and Sephacryl S-1000 (18 × 1.4 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden), respectively. The enzyme solutions were added to the columns and eluted with 0.002 M to 0.1 M sodium acetate buffer (pH 5.2) or doubly distilled water. Fractions of 2 ml (Sephacryl S-200) or 0.6 ml (Sepharose 4B and Sephacryl S-1000) were collected. The void volume (V_o) for Sephacryl S-200 and Sepharose 4B was determined as in [3]. When Sephacryl S-1000 filtration was carried out the void volume was estimated from the elution volume of killed microorganisms, as recommended by the manufacturer. Standard calibration curves for Sephacryl S-200 were constructed as in [11].

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2.4. *Exo-β-glucanase activity determinations*

β-Glucanase activity was assayed as in [7].

2.5. *Polyacrylamide, cellogel and immunoelectrophoresis*

Polyacrylamide gel electrophoresis was performed as described in [9]. Electrophoresis in cellogel (Chemetron, Milano, Italy) was carried out as recommended by the manufacturer. Protein staining was performed according to [12] and exo-1,3-β-D-glucanase activity was tested by cutting the gels into 1 mm slices and incubating them with *p*-nitrophenyl-β-D-glucopyranoside (PNPG). Immunological methods were done as in [7].

2.6. *Glucosidase and mannosidase treatment*

Glucosidase and mannosidase treatments of glucanases were developed by incubating 40 Units of exo-β-glucanase with α-glucosidase (1 Unit), β-glucosidase (10 Units) and α-mannosidase (0.2 Units), in 0.1 M acetate buffer, at 30°C for 12 h.

3. RESULTS

3.1. *Gel exclusion chromatography*

In previous experiments, β-glucanases were subjected to gel exclusion chromatographies with 0.05 to 0.1 M sodium acetate or citrate buffers and, under such conditions, the determined M_r's were in the range of those estimated by analytical polyacrylamide gel electrophoresis. However, when exo-β-glucanases from different species and sources were subjected to gel exclusion chromatographies in a column of Sephacryl S-200 equilibrated with doubly distilled water, the enzymes eluted in the void volume, although their estimated M_rS in 0.1 M sodium acetate buffer were lower than ca. 60 000 (table 1). The self-associating property depended neither on the growth medium nor on the intra- or extracellular origin of the enzymic preparations. It should be pointed out that the exo-β-glucanases partially or totally depleted of their carbohydrate moiety continued exhibiting such an aggregation property.

The self-associated β-glucanases were subjected to filtration through Sepharose 4B and Sephacryl S-1000, but even then they still eluted in the void volume. In view of these results, the M_r of the aggregated exo-β-glucanases must be higher than 6×10^6 but cannot be determined with exactitude.

The tendency towards self-aggregation exhibited by the exo-β-glucanases was lowered by using increasing concentrations of sodium acetate buffer (0.005 to 0.025 M) and was promptly and completely reversed by the addition of 0.025 to 0.1 M sodium acetate buffer (table 2), though it was not affected by 0.1% Triton X-100 and other detergents. The aggregation did not modify the hydrolytic characteristics of the molecule in terms of substrate specificity, K_m values and the like.

Table 1

Self-associating properties of the exo-β-glucanases from different microorganisms

Microorganism	Enymic preparation	Reference	Self-association
S. cerevisiae X_{14}	Purified exo-β-glucanase III	[7]	+
S. cerevisiae X_{14}	Endo H-underglycosylated exo-β-glucanase III	[7]	+
S. cerevisiae ts ^{136}*Me10*	Tunicamycin underglycosylated exo-β-glucanase III	[7]	+
S. cerevisiae S288C	Predominant exo-β-glucanase	[8]	+
S. cerevisiae S288C	Predominant exo-β-glucanase treated with α- and β-glucosidases and α-mannosidase	[8]	+
C. utilis	Exo-β-glucanase	[9]	+
G. lactis	Predominant exo-β-glucanase from cell-free extracts	[10]	+
Kl. phaseolosporus	Purified exo-β-glucanase III	[2]	+

Table 2

Stages of self-aggregation of exo-β-glucanases from different yeast

	Doubly distilled	0.1% Triton X-100	0.005 M Buffer	0.010 M Buffer	0.025 M Buffer	0.1 M Buffer
S. cerevisiae	n	n	3	2	1	1
C. utilis	n	n	3	2	1	1
Kl. phaseolosporus	n	n	3	2	2	1
G. lactis	n	n	3	2	1	1

Arabic numerals refer to the aggregation number taking as reference the smallest functional unit obtained with the ionic strength of 0.1 M sodium acetate buffer

n = non-determined, but $> 10^2$

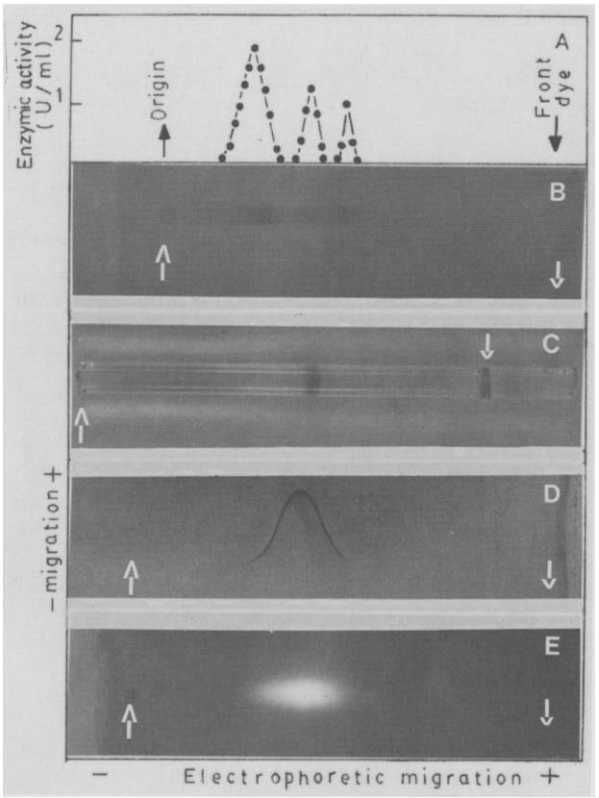

Fig.1. Electrophoretic migration of the exo-β-glucanase III purified from *S. cerevisiae* X_{14} in: cellogel (A,B), enzymic activity on PNPG (A) and protein staining (B); polyacrylamide gels (0.1% SDS), protein staining (C); Agarose gels, enzymic activity on 4′-methyl-umbelliferyl-β-D-glucopyranoside (D); and bidimensional immunoelectrophoresis, protein staining (E).

A number of purified proteins were tested for their ability to aggregate in the presence of doubly distilled water and different results were obtained. Ferritin and thyroglobulin exhibited self-associating properties under the described conditions, whereas ovalbumin and cytochrome *c* did not undergo aggregation.

3.2. *Cellogel, polyacrylamide, agarose and immunoelectrophoresis*

In view of the apparent wide distribution of the self-associating property among β-glucanases some experiments were carried out with one of the glucanases in order to establish the conditions for aggregation to take place. Our attention was focused on the exo-β-glucanase III purified from *S. cerevisiae* X_{14} [7] taking as a reference the migration pattern that the enzyme showed in polyacrylamide gel electrophoresis (0.1% SDS). Under such conditions, the exo-β-glucanases migrated as a single band with an estimated M_r of 61 000. Aliquots of the purified enzyme were subjected to agarose electrophoresis or two-dimensional immunoelectrophoresis; under those conditions the exo-β-glucanase appeared as a single broad band. However, the glycoprotein migrated in a three band pattern in cellogel electrophoresis (fig.1), as detected by protein staining and enzymic activity on PNPG. These fractions with different R_F could be interpreted as intermediate states in aggregation, because the possibility of their being isoenzymes of the exo-β-glucanase, unseparable by the other methods employed, should include the fact that they showed different isoelectric points.

4. DISCUSSION

The results reported in this paper seem to indicate that the exo-β-glucanases from yeasts and perhaps also from filamentous fungi are in fact aggregates of many functional units and are of high M_r. These compounds should dissociate in laboratory conditions due to the ionic strength of the buffers used to maintain the optimal pH value for enzymic activity. In view of the data presented here and of the results previously described, it would be perhaps convenient to reconsider the relative molecular masses estimated for the exo-β-glucanases purified from different sources, because the self-associating properties do not appear to be circumscribed to a single strain or to a small number of yeast species. This phenomenon has also been reported for an hepatic membrane protein responsible for the clearance of serum asialoglycoproteins in rabbits [13].

The proteins must aggregate by means of weak links which are easily broken and dissociated by ionic strength, but which are not affected by Triton X-100 and other detergents. This aggregation does not seem to modify the hydrolytic characteristics of the enzymic molecule. According to the results shown in table 1, the self-associating properties must depend only on the polypeptide fraction of the glycoprotein, because the exo-β-glucanases depleted of their carbohydrate moiety continued to exhibit aggregation. The amino acid composition of the exo-β-glucanase III purified from *S. cerevisiae* X_{14} is very similar to that reported by other workers for different β-glucanases [9,14,15]. These enzymes contain a relevant percentage of acidic amino acids, and other amino acids such as glycine and alanine have been found in high proportion. Most of them are freely solubilised in water except glutamic acid which is only dissolved to a limited extent (8.6 g/l, 25°C), this compound is the second of the amino acids predominant in the hepatic membrane protein responsible for the clearance of serum asialoglycoproteins in the rabbit [13]. Whether or not certain amino acids are responsible for self-aggregation in doubly distilled water is at present not clear but with the data shown one could speculate on the possibility that yeast β-glucanases tend to lower their solubility in aqueous solvents of weak ionic strength.

The possibility that the three fractions of purified exo-β-glucanase III separated in cellogel electrophoresis are isoenzymes rather than states of aggregation is more difficult to accept because if this were so they should exhibit different isoelectric points.

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