Phytochemistry, Vol. 25, No. 2, pp. 359–361, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00

MULTIPLE SUB-UNIT STRUCTURE AND MICROHETEROGENEITY OF α-GALACTOSIDASE I FROM *VICIA FABA* SEEDS

PRAKASH M. DEY, SURBHI NAIK and JOHN B. PRIDHAM

Department of Biochemistry, Royal Holloway College, University of London, Egham Hill, Egham, Surrey, TW20 OEX, U.K.

(*Revised received* 17 *June* 1985)

Key Word Index—*Vicia faba*; Leguminosae; broad bean; isoelectric point; multiple forms; sub-units.

Abstract—Tetrameric α-galactosidase I from *Vicia faba* seeds is dissociated with urea (2.5–5.0 M) into active sub-unit forms. At low urea concentrations dissociation is only apparent if methyl α-D-mannoside is present which may be indicative of the involvement of lectin interactions in subunit aggregation. Chromatofocusing of α-galactosidase I yields multiple tetrameric forms with pI values ranging from 8.75 to 7.35. It is suggested that native α-galactosidase I is a closely related mixture of tetramers resulting from post-translational changes in the enzyme protein.

INTRODUCTION

It is not uncommon to find glycosidases existing as single M_r forms but composed of protein species with different isoelectric points [1–4]. Whether these species are 'artifacts' of extraction and purification procedures is difficult to ascertain although working at low temperatures (*ca* 0°) minimizes this possibility. However, the formation of such artefacts was clearly excluded in the case of β-galactosidase from *Petunia hybrida* which was shown to occur in four isoelectric forms [2]. It must be assumed that the observed multiplicity was, therefore, due to pre- or post-translational changes in the enzyme.

We have demonstrated that α-galactosidase in dormant *Vicia faba* seeds exists in three M_r forms (as determined by sedimentation equilibrium; see ref. [5]) I (M_r 160400 ± 2850) II^1 (M_r 45730 ± 3073) and II^2 (M_r 43390 ± 1409) [5]. All three forms display glucose/mannose specific lectin activity [6, 7]. Enzyme I eluted as a single peak from ion-exchange (CM-cellulose) and gel filtration (Sephadex G-200 and Sephacryl S-200) columns but when examined by SDS-PAGE, a major but microheterogeneous protein band with an overall mobility similar to II^2 was observed [6, 7]. It was suggested that I was a tetramer of II^2; there were also immunological similarities between I and II^2 [7, 8]. This paper describes the separation of various isoelectric forms of α-galactosidase I. We have also been able to dissociate the enzyme into active sub-unit forms.

RESULTS AND DISCUSSION

Dissociation of α-galactosidase I

α-Galactosidase I was prepared according to a method described earlier [7] and attempts were made to dissociate the tetrameric enzyme into active low M_r forms using various experimental conditions. In all experiments M_rs were determined by a gel-filtration method [9, 10]. Treatment of the enzyme (Fig. 1) with varying concentrations of urea (2.5–5.0 M) for 2 hr at pH 5.5 followed by passage through Sephacryl S-200 columns yielded a range of enzymically active dissociation products. With 2.5 M urea (Fig. 1b), a small proportion of enzyme I was converted to a form with an apparent M_r of 80000 which is probably a dimer. With 3.5 M urea, most of enzyme I was dissociated (Fig. 1c) mainly to the dimer and, to a lesser extent, a trimer (M_r *ca* 120000). The profile (Fig. 1d) obtained with 5 M urea implied that a mixture of forms, perhaps monomer, dimer and trimer, had been produced but the resolution was poor. However, the fractions corresponding to the monomeric enzyme (Fig. 1d) were pooled and further analysed by CM-cellulose ion exchange chromatography. A single peak of activity was eluted with a continuous gradient of sodium chloride (Fig. 2b). As expected [7] this peak closely corresponded to the elution profile of a purified sample of α-galactosidase I (Fig. 2a). Moreover, a mixture of authentic II^2 and the gel-filtered enzyme obtained by treatment with 5.0 M urea yielded an identical elution profile (Fig. 2c) to that of pure II^2. It should be noted that the closely related M_r form, α-galactosidase II^1, has a much lower elution volume under identical conditions [7]. This is further proof that enzyme I is composed of sub-units of II^2.

The effect of methyl α-D-mannoside on dissociation of *V. faba* α-galactosidase II was also assessed. Incubation at 30° for 2 hr with 1.5 M urea caused no apparent dissociation of the enzyme and the elution profile was similar to that shown in Fig. 1a. However, when this urea treatment was repeated in the presence of 1 M methyl α-D-mannoside, the enzyme partially dissociated (Fig. 3) to yield an active monomeric form and an enzyme with a lower elution volume corresponding to a dimeric form. Similar results were obtained by incubation with a mixture of 2.5 M urea and 0.5 M methyl α-D-mannoside. 0.5 M or 1.0 M methyl α-D-mannoside alone produced no dissociation of enzyme I. It is conceivable that these results indicate the presence of the normal quaternary bonding forces such as hydrogen bonds but, in addition, monomer aggregation is aided by lectin interactions as previously suggested by Dey *et al.* [7]. Hence, the urea at a relatively low concentration weakens the hydrogen bonding sufficiently for methyl α-D-mannoside to disrupt

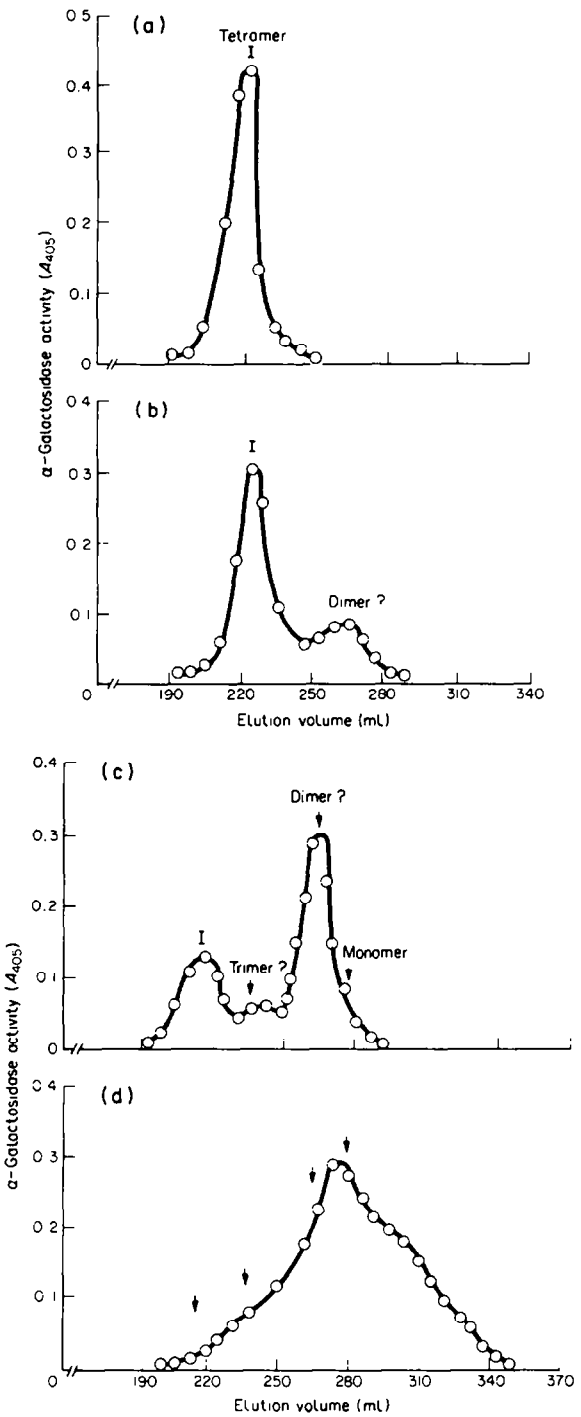

Fig. 1. Treatment of α-galactosidase I from *V. faba* with urea followed by Sephacryl S-200 gel-filtration: untreated enzyme (a), enzyme incubated with 2.5 M urea (b), 3.5 M urea (c) and 5.0 M urea (d). Working details are given in the Experimental section.

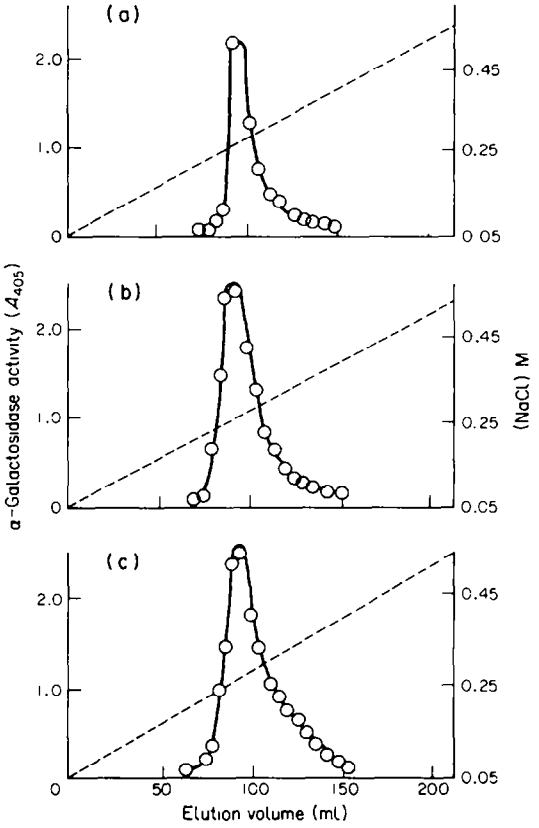

Fig. 2. CM-cellulose chromatography of (a) native α-galactosidase I; (b) monomeric enzyme isolated after treatment with 5.0 M urea (elution volume, 250–290 ml, Fig. 1d); (c) a mixture (1 : 1 with respect to activity) of isolated enzyme as used in (b) and authentic α-galactosidase II[2]. Working details are given in the Experimental section.

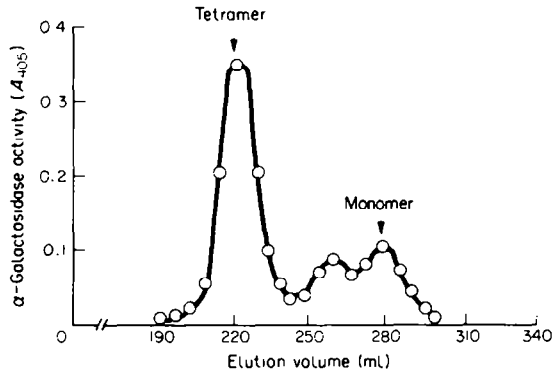

Fig. 3. Sephacryl S-200 gel-filtration profile of *V. faba* α-galactosidase I (tetrameric form) which had been perincubated in a solution containing 1.5 M urea and 1 M methyl α-D-mannoside. Other details are given in the Experimental section.

the lectin interactions by specific hapten competition for lectin receptors.

Isoelectric forms of α-galactosidase I

The monomer components of α-galactosidase I exhibit microheterogeneity when examined by SDS-PAGE [6, 7]; hence, an attempt was made to detect tetramers with minor structural differences. Isoelectric focusing of enzyme I on polyacrylamide gel followed by detection of enzymic activity with 4-methylubelliferyl α-D-galactoside showed the presence of a number of enzyme forms differing in their pI values. The complex separation profile made it difficult to determine the exact number and

relative quantities of the isoelectric forms. It was therefore decided to resolve and quantitatively measure these forms by chromatofocusing.

Following application of the enzyme sample to the chromatofocusing column, elution was carried out with Poly buffer 96 (pH range 9.0–6.0). From the resulting profiles of α-galactosidase activity, protein (E_{280}) and pH, *ca* seven enzymically active peaks can be distinguished which display different pI values ranging from 8.75 to 7.35; 1, pI 8.75; 2, pI 8.35; 3, pI 8.10; 4, pI 7.85; 5, pI 7.65; 6, pI 7.50; 7, pI 7.35). Peaks 1 and 5 appear to be the major forms. There appeared to be no protein peaks without associated enzyme activity.

In separate experiments purified α-galactosidase I was dialysed and gel-filtered through appropriate equilibrated Sephacryl S-200 columns using 0.01 M Tris–HCl buffer, at pH 9, 8 or 7, containing 0.1 M sodium chloride. In all cases the enzyme eluted as the tetrameric form suggesting that the neutral and alkaline pH values encountered by the enzyme on the chromatofocusing column would be unlikely to cause dissociation. Therefore, the peaks 1–7 probably represent different tetramers composed of near identical subunits. Such a phenomenon would be expected, for example, if enzyme I was: (1) derived from monomers with varying degrees of glycosylation (see also ref. [11]) and/or (2) modified by minor post-translational degradation of its polypeptide chains.

In contrast, the tetrameric α-galactosidases from *V. radiata* [12] and *Glycine max* [13] can be dissociated by raising the pH from 4 to 7. This process is reversible. Furthermore, the tetrameric enzymes from *V. radiata* and *Lens culinaris* when subjected to isoelectric focusing exhibit single peaks with pI values of 7.8 [12] and 8.5 [10], respectively.

EXPERIMENTAL

Enzyme assay. α-Galactosidase activity was assayed by using *p*-nitrophenyl α-D-galactoside as the substrate as described earlier [14]. Protein was assayed by the method of ref. [15] with BSA as the calibration standard.

Enzyme purification. Testa free dormant *V. faba* seeds were powdered in a mechanical grinder and α-galactosidase I was isolated and purified as described earlier [7].

Isoelectric focusing. Ready-prepared Ampholine PAG-plates (pH range 3.5–9.5; LKB) were used. The instruments used were LKB 2117 Multiphor cooling plate with some insulating fluid (light paraffin oil). The two electrode strips were saturated with 1 M NaOH and 1 M H_3PO_4, respectively, and placed on either side of the gel connecting to the cathode and the anode tanks. The samples were applied in 2–5 μl droplets (protein 2 mg/ml) directly onto the gel surface, connected to the power supply with the current at 50 mA. The voltage was increased at 10 min intervals, until it reached 1500 V. The experiment was run for 1.5 hr. The enzyme activity was detected by the use of fluorescent substrate, 4-methylumbelliferyl-α-D-galactoside. The gels were covered with a soln of substrate (5 mg/10 ml), McIlvaine buffer, pH 5.5, and incubated for 5–10 min at room temp. The enzyme activity was located using UV light and the gels were photographed using a Polaroid camera.

Chromatofocusing. The manufacturer's (Pharmacia) instructions for packing and running the column were followed. The gel and the buffers used were degassed immediately before use. The column (1 cm × 15 cm) was packed and equilibrated with 0.02 M ethanolamine–acetic acid buffer, pH 9.4, the enzyme sample was dialysed against Polybuffer 96, pH 6 and applied to the column after running on 5 ml of this buffer to prevent the sample proteins from being exposed to extremes of pH. The column was then eluted with 250 ml of the buffer at a flow rate of 30–40 ml/hr collecting 2 ml fractions. The pH of each fraction was monitored and A_{280} and α-galactosidase activity was determined.

Dissociation of α-galactosidase I. To α-galactosidase I (395 nkat/ml; 2 ml) in McIlvaine buffer, pH 5.5, solid urea was added to final concns of 1.5, 2.5, 3.5 or 5.0 M. The samples were incubated at 30° for 2 hr and then applied separately to calibrated Sephacryl S-200 columns (2.5 cm × 90 cm). Elution was carried out with McIlvaine buffer, pH 5.5 and 3 ml fractions were collected and assayed for enzyme activity.

The enzyme fractions corresponding to the monomeric form (*M, ca* 40000) were pooled and subjected to CM-cellulose chromatography. In a separate experiment a mixture of the pooled fractions and α-galactosidase II[2] isolated as described earlier [7] (~ 60 nkat of each) was subjected to CM-cellulose chromatography and the eluted fractions assayed for enzyme activity.

In addition to treating α-galactosidase I with urea alone, the effect of methyl α-D-mannoside (1 M) in the presence of urea (1.5 M) was examined following the incubation and gel-filtration conditions as described above.

CM-Cellulose chromatography. CM-52 was prepared as described in the manufacturer's instruction sheet and equilibrated with McIlvaine buffer, pH 3.5, and a column (1 × 14 cm) packed at a flow rate of 20 ml/hr. The enzyme preparation was dialysed against this buffer and applied to the column followed by elution with the same buffer until the absorbance at 280 nm was negligible. The bound enzyme was then eluted with a linear NaCl gradient prepared from 0.05 M NaCl (100 ml) and 0.5 M NaCl (100 ml) both in McIlvaine buffer, pH 3.5. Fractions (2 ml) were collected at a flow rate of 15 ml/hr and measured for A_{280} and enzymic activity.

Acknowledgement—We are indebted to the Central Research Fund of the University of London for an equipment grant. S.N. received financial support from the British Council.

REFERENCES

1. Lundbald, G., Huldt, G., Elander, M., Lind, J. and Slettengren, K. (1981) *Comp. Biochem. Physiol.* **B68**, 71.
2. Komp, M. and Hess, D. (1981) *Phytochemistry* **20**, 973.
3. Peruffo, A. D. B., Renosto, F. and Pallavicini, C. (1978) *Planta* **142**, 195.
4. Dey, P. M. and Del Campillo, E. (1984) *Adv. Enzymol.* **56**, 141.
5. Pridham, J. B. and Dey, P. M. (1974) in *Plant Carbohydrate Biochemistry* (Pridham, J. B., ed.) pp. 83–96. Academic Press, London.
6. Dey, P. M., Naik, S. and Pridham, J. B. (1982) *FEBS Letters* **150**, 233.
7. Dey, P. M., Pridham, J. B. and Sumar, N. (1982) *Phytochemistry* **21**, 2195.
8. Dey, P. M., Hustler, M. J., Pridham, J. B. and Sumar, N. (1982) *Phytochemistry* **21**, 1557.
9. Andrews, P. (1964) *Biochem. J.* **91**, 222.
10. Dey, P. M., Del Campillo, E. and Pontlezica, R. (1983) *J. Biol. Chem.* **258**, 923.
11. Hatton, M., Marz, L. and Regoeczi, E. (1983) *Trends Biochem. Sci.* **8**, 287.
12. Del Campillo, E., Shannon, L. M. and Hankins, C. N. (1981) *J. Biol. Chem.* **256**, 7177.
13. Del Campillo, E. and Shannon, L. M. (1982) *Plant Physiol.* **69**, 628.
14. Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **113**, 49.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.