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SHORT REPORTS

IDENTIFICATION OF CANAVALIN AS A PROTEOLYTICALLY MODIFIED FORM OF JACK BEAN α-D-MANNOSIDASE

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Key Word Index—*Canavalis ensiformis*; canavalin; α-D-mannosidase; X-ray crystallography.

Abstract—The structure to 3.0 Å resolution of the protein canavalin from Jack beans has been determined by conventional X-ray crystallographic techniques. We present evidence that canavalin is a proteolytically modified form of the enzyme α-D-mannosidase. This is based on the facts that the purified precursor protein possesses substantial α-D-mannosidase activity; it comigrates on SDS-PAGE with Jack bean α-D-mannosidase prepared by other means; and its oligomer molecular weight and Zn^{2+} content are the same as those of Jack bean α-D-mannosidase.

INTRODUCTION

The proteins concanavalin A, concanavalin B and canavalin were isolated from the Jack bean seed (*Canavalis ensiformis*) by Sumner in 1919, the first two in crystalline form and the latter as an amorphous precipitate [1]. In 1936 Sumner and Howell discovered that canavalin could be crystallized if exposed to trypsin or microbial proteases [2]. The physiological role of the three proteins has not yet been established, though concanavalin A has found wide use as a lectin and mitogen, and has been the subject of extensive study and characterization [3]. Concanavalin A and canavalin are the two most prominent proteins present in the Jack bean seed, each comprising about 2.5% of the total soluble protein [4].

Five years ago we undertook a three-dimensional structure analysis of concanavalin B and canavalin using single crystal X-ray diffraction techniques. To facilitate interpretation of our crystallographic results, we conducted a parallel biochemical characterization of the two proteins and attempted to deduce their function in the developing seed [5]. Although our studies on concanavalin B are as yet not complete, we have produced a 3.0 Å resolution electron density map of canavalin from which the course of the polypeptide backbone has been derived [6]. Almost simultaneous with the calculation of our model structure from X-ray data, we acquired a substantial body of evidence suggesting that the protein canavalin is a proteolytically modified form of the enzyme α-D-mannosidase.

RESULTS

Canavalin is a hexamer of 126 000 MW composed of three pairs of similar but non-identical subunits related by a perfect three-fold axis of symmetry and pseudo dyad axes, thereby endowing the molecule with a high degree of 32 point group symmetry. One of each pair of subunits is derived from the amino terminal half of the precursor polypeptide of MW 42 000 and the other from its carboxy terminal half. Thus the crystallographic evidence indicates that the precursor polypeptide is a tandem duplicate and is structurally redundant [6]. The pathway for formation of canavalin from the precursor molecule, which is also a hexamer of six identical 42 000 MW subunits, as deduced from our electrophoretic and crystallographic studies, is shown in Fig. 1.

We have obtained the following evidence indicating that the canavalin precursor, which we have in the past referred to as precanavalin, and which exists in the seed prior to proteolytic modification, is the enzyme α-D-mannosidase.

(a) Precanavalin co-migrates with commercially available Jack bean α-D-mannosidase on gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE), both demonstrating a subunit MW of 42 000.

(b) Precanavalin demonstrates a specific activity using *p*-nitrophenyl-α-D-mannoside as substrate of *ca* 30% that of the commercial enzyme prepared by the method of Li and Li [7]. That there is reduced activity is not surprising in light of the fact that

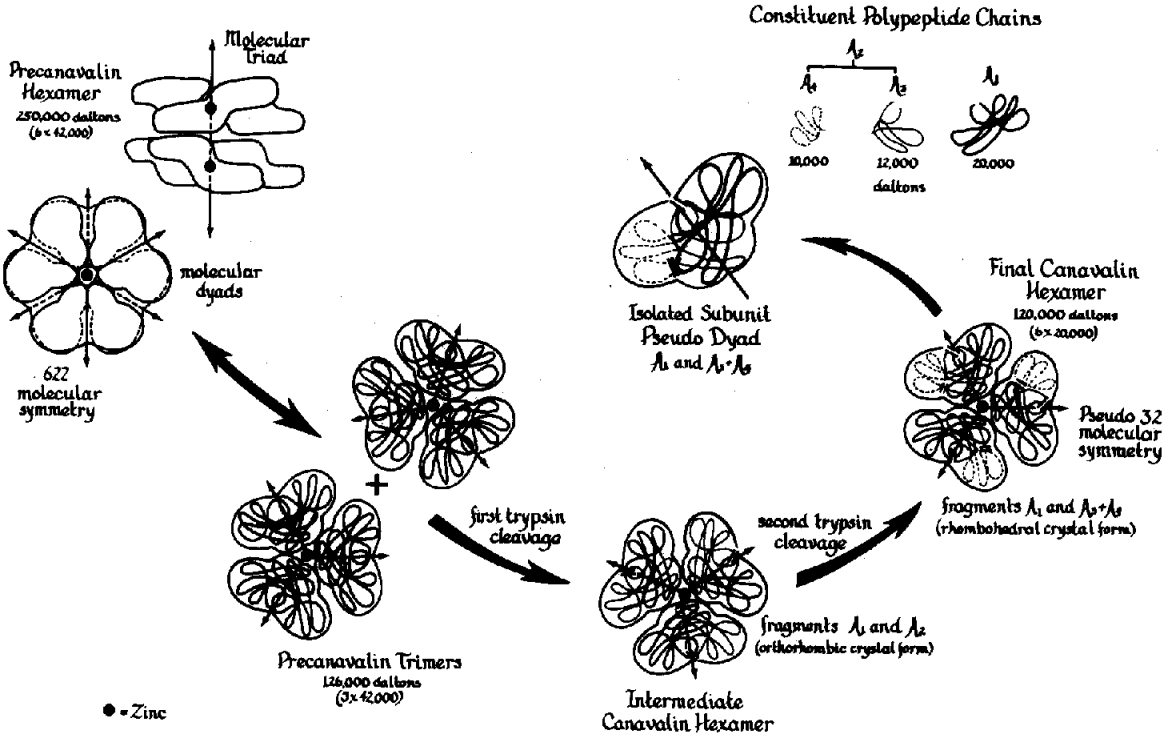

Fig. 1. Conversion of precanavalin to canavalin.

Jack bean α-D-mannosidase is known to be an extremely labile enzyme [8–10] and that our purification procedure included several steps known to have an adverse effect on the enzyme's activity. In addition, 5 times recrystallized and electrophoretically pure canavalin, the proteolytically modified form, still showed a specific activity 15% that of the commercial enzyme.

(c) The oligomer MW of precanavalin was found by gel filtration to be approximately 200 000. This is the same as has been reported for Jack bean α-D-mannosidase prepared by other means [7, 10].

(d) Precanavalin was found to contain two zinc ions per mol. This is the same zinc content previously reported for Jack bean α-D-mannosidase [10].

(e) The purified precursor protein was crystallized from 50% saturated ammonium sulfate to give crystals of the same morphology as was obtained by others under the same conditions but using enzyme prepared by other means [7].

We hasten to emphasize that our preparations of both canavalin and the precursor protein which we contend is α-D-mannosidase were extremely pure on SDS-PAGE showing no observable contaminants for the former and no more than a few per cent for the latter and that both were crystallized. Thus, we do not believe that contaminating proteins could be responsible for the observed activity or the other properties we have noted. It further seems unlikely that our precanavalin would co-migrate with α-D-mannosidase both as an oligomer and as a subunit, and have in addition the same molar ratio of protein to zinc. Thus we feel reasonably safe in assuming that the protein molecule, whose structure we have solved by X-ray crystallographic analysis and which is known in the literature as canavalin, is indeed a modified form of the enzyme α-D-mannosidase.

If Jack bean seeds are extracted with a 2% SDS solution so as to retrieve virtually all of the soluble protein and this extract is examined by SDS-PAGE, α-D-mannosidase is seen to be the major seed protein present. The second most abundant protein in the seeds is concanavalin A. This protein, perhaps not coincidentally, is also a mannose-specific oligosaccharide binding protein as is α-D-mannosidase. Because these two proteins comprise such a vast reservoir of the seeds total supply, it has been widely assumed that they are simply storage proteins. In light of the structural and functional complexity of concanavalin A, and our evidence identifying precanavalin with α-D-mannosidase, we believe this view is no longer tenable.

EXPERIMENTAL

Preparation and crystallization of precanavalin was according to Sumner [1] and canavalin by Sumner and Howell [2] as modified by McPherson and Spencer [11]. Commercial α-D-mannosidase was purchased from Sigma Co. and Boehringer–Mannheim Co. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [12] using a discontinuous Tris–glycine buffer system. Slab gels were gradients of 5–15% acrylamide and were stained with Coomassie blue according to Weber and Osborn [13]. α-D-Mannosidase assays were as described by Li and Li [7] using *p*-nitrophenyl-α-D-mannoside as substrate and monitoring the absorbance change at 400 nm after

30 min and development at alkaline pH. The sp. act. was computed based on a standard definition that 1 unit of enzyme activity produces 1.0 μmol p-nitrophenol per min at 25°. Protein determinations were by Lowry [14]. MWs were determined by gel filtration on a column 1×100 cm packed with Sephadex G-200 equilibrated with 0.05 M Tris–HCl at pH 7.5. A series of standard proteins were also run to calibrate the column which was continuously monitored. The metal ion content of the proteins was determined by Galbraith Laboratories, Knoxville, TN using atomic absorption spectrophotometry. The 3-dimensional structure of the molecule was determined by conventional X-ray diffraction techniques using isomorphous replacement with 5 heavy atom derivatives. The details of this analysis will be published elsewhere [5].

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N-BENZOYLASPARTATE AND *N*-PHENYLACETYLASPARTATE FROM PEA SEEDS*

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Key Word Index—*Pisum sativum*; Leguminosae; pea; amino acid; acyl amino acid; *N*-phenylacetylaspartate; *N*-benzoylaspartate.

INTRODUCTION

In our continuing study of the metabolism of developing fruits and seeds of the G2 line of peas, we have isolated and identified two naturally occurring *N*-acyl amino acids which have not been reported previously. These compounds are the aromatic amides of aspartic acid, namely, *N*-benzoylaspartate and its homologue *N*-phenylacetylaspartate.

A variety of acyl amino acids have been found in living systems; *N*-acetylaspartate is present in mammalian brain tissue [1], hippuric acid (*N*-benzoylglycine) is a normal constituent of human urine [2] and *N*-phenylacetylglutamine has been found in cow's milk [3]. In plants, the metabolism of exogenously supplied benzyl alcohol and benzoic acid to *N*-benzoylaspartate has been reported in barley [4], and in auxin-treated pea stem segments [5]. The following paper describes the isolation and identification of these substances by chemical tests and MS analysis. The results were confirmed by MS of the chemically synthesized derivatives.

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RESULTS AND DISCUSSION

Developing fruits were enclosed in glass chambers in the presence of $^{14}CO_2$ for 24 hr. The acidic EtOAc-soluble fraction of the 80% MeOH extract was chromatographed by Si gel HPLC in $CHCl_3$–MeOH–HOAc (70:30:1). The radioactive zone co-chromatographing with abscisic acid (ABA) was removed and chromatographed in $CHCl_3$–MeOH–HOAc (90:10:1). The major radioactive zone (R_f 0.125) was clearly separated from ABA (R_f 0.46) in this system.

Chemical tests for functional groups indicated that