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Note

Purification of intact concanavalin A in tetramers by isoelectric focusing

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The availability of homogeneous material is a prerequisite for the unequivocal interpretation and quantitative evaluation of any test in which it is impossible to sort out the contributions from the various reactants, if there were several, and only average effects can be recorded. Our group has been interested for some time in studying the physicochemical and thermodynamic properties of concanavalin A (Con A) [1–3]. However, it is known that the normal metabolism of the *Canavalia ensiformis* seed causes post-translational modification of the primary gene product, a 27-kDa peptide chain [4]. Modification of the Con A structure may also occur during purification and storage of the protein. The two resulting fragments do not dissociate but form nicked protomers [4], in contrast to intact forms. Moreover, the structure of Con A is oligomeric; at low pH (<5.5) it exists as a dimer but at high pH (>7.2) it is a tetramer [4,5]. Procedures for the purification of homogeneous material, i.e., of intact monomers, have been described. These rely upon size fractionation in the presence of strongly denaturing and dissociating additives [urea, guanidine hydrochloride, sodium dodecyl sul-

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phate (SDS)] by either conventional [6] or high-performance liquid [7] chromatography. Purification of tetramers of intact monomers only has also been obtained by affinity chromatography on dextran resins, with D-glucose as the eluent [8].

In this paper we describe a novel approach, based on charge fractionation, in which tetramers are resolved, under native conditions, by preparative isoelectric focusing (IEF) on a granulated gel bed.

EXPERIMENTAL

Materials

Acrylamide, N,N′-methylenebisacrylamide (Bis) and Bio-Gel P-60 were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Ampholine carrier ampholites were purchased from LKB (Bromma, Sweden), Con A (co, LK01093), Silane A174, p*I* and molecular weight standards from Pharmacia (Uppsala, Sweden), Sepraphore III acetate strips from Gelman (Ann Harbor, MI, U.S.A.) and PM30 ultrafiltration membranes from Amicon (Denvers, MA, U.S.A.). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

Preparation of Con A

Aliquots of lyophilized Con A isolated from jack bean *C. ensiformis* and certified as 98% pure by the supplier were dissolved in distilled water containing 100 m*M* $CaCl_2$–$MnCl_2$ before further purification by IEF.

Analytical IEF

IEF was performed on ultra-thin (240 μm) polyacrylamide slabs (total monomer concentration, $T=7\%$; relative percentage of cross-linker, $C=4\%$) (10×10 cm) supported by silanized glass (coated with 0.1% methacryloxypropyltrimethoxysilane in acetone). The gels contained 12% (v/v) glycerol and 1.9% (w/v) Ampholine in the pH range 7–11. The course of the gradient was evaluated both by reference to six marker proteins (see *Materials*) and with a surface electrode. The gels were pre-run at 500 V for 1 h and run at 13 W and 2000 V for 6 h at 10°C. The anolyte was 0.2 *M* H_3PO_4, the catholyte was 0.2 *M* NaOH and 50 μg of proteins were applied per lane on filter paper pads. The focused proteins were stained with silver nitrate according to Merril et al. [9]. After fixation in 12% (w/v) trichloroacetic acid (TCA) for 20 min, the gels were rinsed three times for 10 min in 10% ethaol–5% acetic acid. They were incubated for 10 min in 3.4 m*M* $K_2Cr_2O_7$–3.2 m*M* HNO_3 in the dark, then for 30 min in 10 m*M* $AgNO_3$, under uniform high-intensity illumination. The developer contained 0.28 *M* $NaHCO_3$ and 2% (v/v) formaldehyde, and 1% acetic acid was used to stop the reaction. The gels were allowed to dry after exaustive rinsing with water.

Preparative IEF on granulated gels

Preparative IEF in layers of granulated gels was performed according to Frey and Radola [10]. Bio-Gel P-60 was washed with 20 volumes of distilled water and mixed with 1.5% (v/v) Ampholine (pH 7–9) and 0.5% of Ampholine (pH

9–11). Layers (1.5-mm thick) were obtained by spreading the gel slurry over a glass plate with the aid of a glass rod and by drying the surface with a fan until irregular 1–2-mm fissures appeared at the edges. In our experiments, between 50 and 200 mg of proteins were mixed with the gel suspension and separated per run. The electrode strips were soaked in 0.1 *M* NaOH (cathode) and 0.1 *M* H_3PO_4 (anode). Focusing was carried out at 10°C at 300 V for 3 h, then at 2000 V for 21 h. The resolved bands were printed on to cellulose acetate strips and stained with 1% (w/v) Ponceau Red in 10% (w/v) TCA. The protein was recovered from the gel by transferring the various fractions into syringes and eluting with water. The solutions were then concentrated by ultrafiltration and dialysed against 1 m*M* acetate buffer (pH 4.7) containing 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. The recovery was checked using the Coomassie G250 protein binding assay [11].

Titration curves

Electrophoretic titration curves were run according to Righetti and Giannazza [12] in polyacrylamide gels (15×15×0.6 cm) with $T=5\%$, $C=4\%$ and containing 2% (v/v) Ampholine (pH 3.5–10.0). The carrier ampholytes were focused at 10°C by delivering a constant power of 10 W until the gel conductivity decreased to 10 mA. The sample (100 μg) was then applied in the central trench and electrophoretic migration across the pH gradient was started perpendicular to the first direction at 600 V. The time allowed for the electrophoresis in the second dimension was 33 min. Experiments were carried out in the presence of 4 *M* urea. The pH gradient was measured on 1 cm wide gel slices, cut 6 cm from the central trough and eluted with 10 m*M* KCl. The protein pattern was stained with Coomassie G250 [13].

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in vertical slabs (140×160×0.75 mm) with the Protean Cell apparatus (Bio-Rad Labs.) using a polyacrylamide matrix with $T=15\%$ and $C=2.6\%$. The discontinuous buffer system was prepared according to Laemmli [14]. The gels were run for 6 h at 150 V and 20 mA. The slabs were fixed for 1 h in methanol–acetic acid–water (5:2:5, v/v/v), then stained overnight in 0.1% Coomassie Blue R250 in the same solvent. The background was destained with 30% methanol–10% acetic acid.

Circular dichroism (CD)

The CD spectra were recorded with a JASCO J500 A spectropolarimeter at room temperature using cuvettes of 0.1 cm path length. The instrument was calibrated by using *d*(+)-10-camphorsulphonic acid, which shows a positive CD band at 290.5 nm with a $\Delta\delta$ of +2.36 l mol^{-1} cm^{-1} (the concentration was determined by using $\epsilon=34.5$ l mol^{-1} cm^{-1} at 285 as the molar absorptivity of the anhydrous form).

Scanning densitometry

An LKB UltraScan XL laser densitometer was used for scanning densitometry of the SDS gel plates.

RESULTS

Fig. 1 shows the crude Con A components as resolved by analytical IEF on polyacrylamide gel (Fig. 1A) and on the electrophoretic titration curve (electrophoresis across a stationary pH gradient, Fig. 1B). In Fig. 1A the major components are marked as C1 to C4, and their p*I* values are listed along the pH scale.

The same labels (C1 to C4) also specify the fractions collected after preparative IEF on Bio-Gel layers, as detected by the composition of the purified fractions. Figs. 2 and 3 show the composition of the purified fractions. In Fig. 2 the refocalization pattern of the four purified isoforms by equilibrium IEF is shown, and Fig. 3 corresponds to their pattern on SDS electrophoresis. Table I gives the amount of each isoform calculated from their size composition.

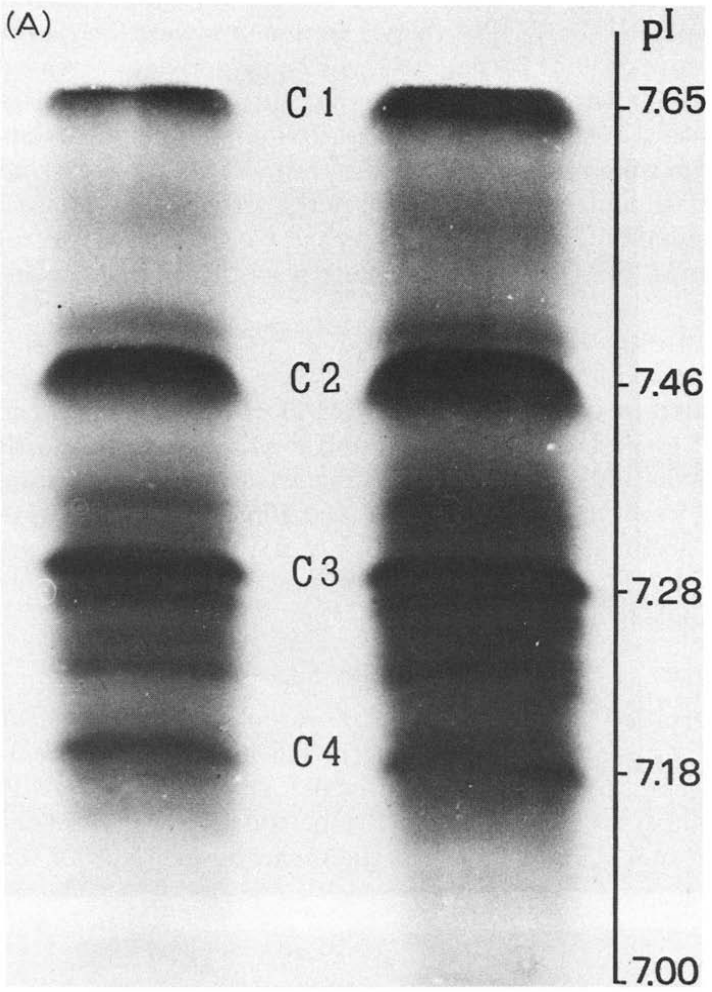

Fig. 1.

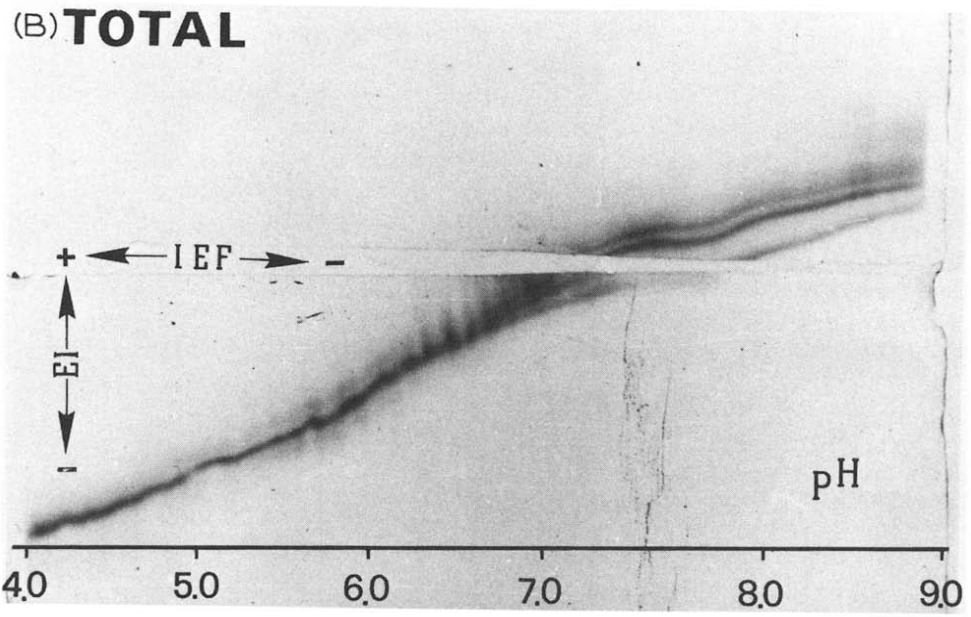

Fig. 1. Characterization of the starting material for Con A fractionation: (A) IEF with a pH 7-10 gradient; (B) electrophoretic titration curve across a stationary pH gradient in the range 3-10 for the same sample of crude Con A purchased from Pharmacia.

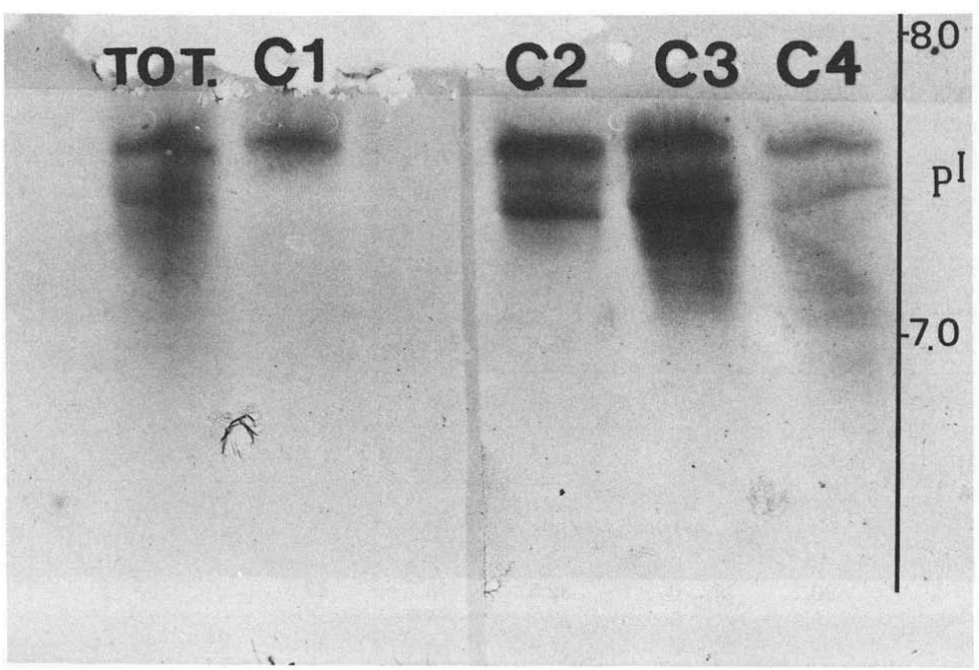

Fig. 2. Characterization of the purified Con A fractions (from preparative IEF) by analytical IEF.

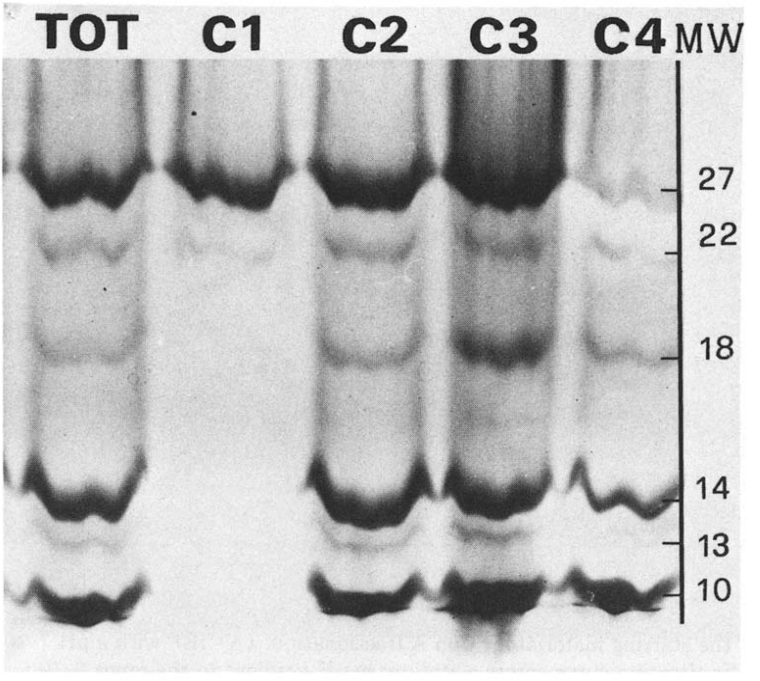

Fig. 3. SDS electrophoresis of fractions C1 to C4 in comparison with the starting material. Densitometry of the stained SDS pattern gave the following ratios for the intact subunit (MW = 27 000; C1, 94%; C2, 34%; C3, 25%; C4, 13% (see Table I). The gel was overloaded with protein sample in order to obtain good visualization of minor components.

Fraction C1 is found to be considerably homogeneous according to size, and under denaturing conditions only the 27 000 molecular mass band corresponding to the intact protomer (96.1%) and a small contamination with a 22 000 fragment (5.9%) are observed. The good degree of homogeneity of C1 is also dem-

TABLE I

SIZE COMPOSITION OF CON A ISOFORMS CALCULATED AS A PERCENTAGE OF CRUDE MATERIAL

Molecular mass ($kDa \times 10^3$)	Con A isoforms (% of total)				
	Crude	C1	C2	C3	C4
27	22.8	94.1	34.1	25.1	13
22	9.8	5.9	8	6.6	9
18	0	0	0	10	16
14	30.7	0	32.5	15.7	17
13	8.3	0	9	3.8	5
10	28.4	0	16.4	38.8	40

onstrated by analytical IEF (Fig. 2). Fractions C2 to C4, in contrast, are heterogeneous with respect to both charge and size, containing significant amounts of 10 000–14 000 peptides and smaller amounts of the 18 000–22 000 fractions. On refocalization, C2 to C4 reproduced their original p*I* values but discrete amounts of material with the p*I* of C1 were also observed. Aliquots from all fractions were also analysed by CD. The spectra (not shown) were qualitatively identical (with θ_{max} at 196 and θ_{min} at 224 nm) and quantitatively very similar, after correction for the different protein concentrations of the various solutions.

DISCUSSION

The SDS-PAGE analysis of the purified components of Con A after preparative flat-bed IEF in granulated gel layers demonstrates that the fraction with p$I = 7.65$ (C1) has a good degree of homogeneity and contains only intact (non-nicked) protomers. This finding suggests the adoption of IEF for small- and medium-scale preparative purposes for the purification of homogeneous Con A lots from crude commercial batches. As recognized since it was first proposed [15], the use of granulated gels as an anticonvective support for IEF allows (i) a higher sample load than with density gradient columns, (ii) a clear identification of the band of interest and the possibility of correcting for their distortion, if any, through the printing of the whole protein pattern and (iii) an easy, fast and usually quantitative recovery of the applied material, typically with only 1:3 dilution from the isoelectric state.

More difficult, however, is the attribution of fractions C2 to C4, all of which contain a variable combination of intact and/or nicked forms, according to SDS-PAGE. A major puzzle is the number of resolved components, although the purity of the starting material is certified by the producer to exceed 98%.

Apart from fraction C1 that maintains a constant p*I*, all other isobands from C2 to C4 produce on refocalization other bands in addition to their original band. This observation confirms that protomer and nicked forms are in equilibrium and when the former is absent (as in the case of C2 to C4) the latter tends to aggregate [16].

The pH dependence of the electrophoretic mobility of the components of crude Con A is shown in Fig. 1B: a single curve is visible in the acidic pH region, whereas above pH ca. 6.5–7 three curves diverge to cross the p*I* plane between pH 7 and 8 and then run virtually parallel in the alkaline region. The singleness of the migration below pH 5 indicates that below this pH all the isofractions share the same electrical charge, in agreement with the concept that below this pH Con A dissociates to form stable dimers. The number of diverging lines in the neutral region fits with the number of the major isobands separated by equilibrium IEF, apart from an increased sensitivity of the latter technique due to silver staining. The pH of the region at which level the three lines begin to diverge during the electrophoretic titration experiment suggests a dependence of the phenomenon on histidine residues, which are the unique amino acid to be titrated in this region. As it appears from SDS-PAGE that the most alkaline isoband corresponds to intact protomer, it seems reasonable to conclude that a different number of res-

idues or pK shifts occur when the protein aggregates to form intact tetramers [17]. These changes could be produced by variation in the hydrophobicity of the amino acid environment due to conformational adaptation.

In conclusion, the use of preparative IEF in granulated Bio-Gel layers allows the purification of an intact Con A protomer characterized by the absence of nicked forms of the protein. With the aid of this technique we have obtained highly purified samples of Con A, which is a fundamental prerequisite for studies on the physicochemical and thermodynamic characteristic of this protein.

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