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Structural and electron-microscopic studies of jacalin from jackfruit (*Artocarpus integrifolia*) show that this lectin is a 65 kDa tetramer

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The 133-amino-acid sequences of the α-subunit of jacalin (a lectin from *Artocarpus integrifolia*) and of the slightly larger α′-subunit were determined. The α′- and α-subunits, in the approximate ratio of 1:3, were found to be virtually identical in their primary structures, except for one valine for isoleucine substitution at position 113. Although both α′- and α-chains were glycosylated, the extent of glycosylation in the α′-chain was much greater than that in the α-subunit. In the α′-polypeptide, all molecules contained an *N*-linked oligosaccharide at position 74 and some contained sugar at position 43. The α-and α′-subunits were found to be strongly non-covalently associated with three distinct β-subunits containing 20 amino acids each. Electron-microscopic visualization of native jacalin disclosed a structure composed of four α-type subunits with a clear-cut 4-fold symmetry. Analytical-ultracentrifugation studies of jacalin revealed an average molecular mass of 65 kDa, a value compatible with a tetrameric structure of the α(α′)-subunits. The recalculated number of sugar-binding sites per jacalin molecule, given a molecular mass of 65 kDa, would yield 0.8 sites per α(α′)-promoter, i.e. about twice the value previously determined [Appukutan & Basu (1985) FEBS Lett. **180**, 331–334; Ahmed & Chatterjee (1989) J. Biol. Chem. **264**, 9365–9372].

INTRODUCTION

Lectins are considered to be powerful tools in immunochemistry and cellular immunology, although they are proteins of non-immune origin and widely present in plants and animals (Sharon & Lis, 1989). They were first described as precipitating agents for certain serum glycoproteins (Stillmark, 1889) and they agglutinate various cell types, including erythrocytes, leucocytes, tumour cells, bacteria and viruses, a property that can be inhibited by specific oligosaccharides, suggesting that lectins specifically recognize various saccharide structures.

Seeds of the jackfruit *Artocarpus integrifolia* contain a lectin named jacalin which is, *in vitro*, a potent polyclonal activator for human lymphocytes, mostly T cells (Bunn-Moreno & Campos-Neto, 1981) but does not appear to induce B lymphocyte differentiation (Aucouturier *et al.*, 1989). Furthermore, jacalin specifically binds to the subclass 1 of IgA (including monomers, polymers, polyclonal and secretory IgA_1) but not to IgG and IgM (Roque-Barreira & Campos-Neto, 1985; Aucouturier *et al.*, 1987, 1988; Hagiwara *et al.*, 1988). Jacalin was also reported to bind to C1 inhibitor (Hiemstra *et al.*, 1987). The weak interaction of this lectin with IgD and IgA_2 of both allotypes is still a matter of controversy (Aucouturier *et al.*, 1989; Pineau *et al.*, 1991). Selective interaction of proteins with jacalin was attributed to their content of multiple *O*-linked oligosaccharides (Baenziger, 1984). Thus insolubilized jacalin offers a useful tool as an afinity matrix for the purification of various proteins bearing serine- or threonine-*O*-linked sugars (Hortin & Trimpe, 1990). Jacalin has a high affinity for α-galactopyranosides with the structure β-D-Gal-(1→3)-β-D-GalNAc (Sastry *et al.*, 1986; Hagiwara *et al.*, 1988). Such a galactose-containing disaccharide ligand is part of the Thomsen–Friedenreich antigen (T-antigen), a tumour-associated antigen of non-oncofetal origin (Springer & Dessai, 1975). Jacalin should prove a valuable probe for studying the expression of the T-antigen on cell surfaces.

Although this lectin has been extensively studied with respect to its sugar-binding specificity and size of its combining site (Roque-Barreira & Campos-Neto, 1985; Hagiwara *et al.*, 1988; Aucouturier *et al.*, 1989; Ahmed & Chatterjee, 1989), its structural properties have not been fully established; in particular, its quaternary structure is still controversial. According to Appukutan & Basu (1985), Ahmed & Chatterjee (1989) and Aucouturier *et al.* (1989), jacalin behaves as a homotetramer with a molecular mass varying from 39.5 kDa to about 54 kDa. Such tetrameric structures would consist of three unglycosylated α-subunits of 12 kDa each non-covalently associated with one glycosylated α′-subunit of 15 kDa (Aucouturier *et al.*, 1989). Young *et al.* (1989) unravelled three forms of a β-polypeptide of 20 residues, each non-covalently attached to the α-subunits, and proposed that, with the β-chains included, an $\alpha_3\beta_3$ trimer would be 43 kDa, i.e. a plausible alternative to the tetrameric structure. Preliminary X-ray-diffraction studies (Dhanaraj *et al.*, 1988) provide evidence that crystal forms of jacalin contain eight tetrameric molecules (molecular mass approx. 40 kDa) in the unit cell.

These discrepancies and the knowledge of the sequence of the α-subunit published by Young *et al.* (1991) prompted us to examine the relative spatial arrangement of the large subunits in native jacalin by negative-staining electron microscopy, to determine the average molecular mass from sedimentation data and to unravel the structural similarity between α- and α′-chains.

MATERIALS AND METHODS

Reagents

Artocarpus integrifolia seeds, collected in the Indian Ocean island La Réunion, were kindly provided by Dr. Aucouturier, Poitier, France. Minileak beads were purchased from Kem-en-Tek Biotechnology Corp., Copenhagen, Denmark; melibiose was from Sigma, St Louis, MO, U.S.A. Acrylamide, piperazine diacrylamide, urea, poly(vinylidene difluoride) (PVDF) and nitrocellulose membranes were purchased from Bio-Rad Labora-

Abbreviations used: PAS, periodic acid–Schiff; PTH, phenylthiohydantoin; PVDF, poly(vinylidene difluoride).

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tories, Richmond, CA, U.S.A. The glycan-detection kit, chymotrypsin, trypsin and endoproteinase Glu-C were from Boehringer, Mannheim, Germany. Solvents for h.p.l.c. were from Merck, Darmstadt, Germany. Water used for the preparation of buffers were obtained from a Milli-Q water system (Millipore Corp., Bedford, MA, U.S.A.). All other chemicals were analytical grade.

Purification of jacalin subunits and their derived peptides

Jacalin purification. The jacalin crude extract was prepared and prepurified on a DEAE-trisacryl (IBF, Villeneuve-la-Garenne, France) column as described by Aucouturier *et al.* (1987). The three eluted fractions, designated F1, F2 and F3, were repurified by affinity chromatography on Minileak–melibiose conjugate for which jacalin has a high affinity (Hagiwara *et al.*, 1988), with 0.8 M-galactose in 150 mM-Tris/HCl buffer, pH 8.6, as the elution buffer. Each fraction appeared to be pure, when assessed by SDS/PAGE, and active, when assessed by Ouchterlony analysis with various human myeloma IgA_1 preparations. Alternatively, the jacalin crude extract was prepurified on a DEAE-cellulose column (Capon *et al.*, 1990). Material in the void volume was collected and rechromatographed on Minileak–melibiose. The constituent β-, α- and α'-chains of the F2 fraction (from the DEAE-trisacryl column) were separated on a C4 reversed-phase column by the method of Young *et al.* (1989). In other experiments, the α- and α'-subunits of the F2 fraction (1.6 mg portions) were separated on preparative SDS-containing mini-gels that had been cast as described by either Laemmli (1970) or Schägger & von Jagow (1987). In both types of gel, bisacrylamide was replaced by piperazine diacrylamide. After electrophoresis, the proteins were isolated by electroelution using the procedure described in Hughes *et al.* (1990).

Enzymic digestion of α- and α'-subunits. This was performed, after extraction of SDS, essentially as described by Hughes *et al.* (1990) except that digestion buffers contained urea (4 M for trypsin and chymotrypsin, 2 M for endoproteinase Glu-C). When the resulting peptides were to be separated by SDS/PAGE and electroblotting (and not by reversed-phase h.p.l.c.), the digestions were made on non-SDS-extracted material in the same digestion buffers except that urea was replaced by SDS (at a final concentration of 0.1%).

Chemical cleavage of α- and α'-subunits. CNBr digestion and asparaginyl-glycyl bond cleavage by hydroxylamine were performed on electroeluted and SDS-extracted α- and α'-chains by the methods described by Findlay & Geisow (1989).

Chemical modification of α- and α'-subunits. Succinylation was performed as described by Findlay & Geisow (1989). Modified polypeptides were then digested with trypsin or endoproteinase Glu-C. The resulting peptides were separated by reversed-phase h.p.l.c. or SDS/PAGE followed by electroblotting.

Analytical procedures

Purification of peptides by reversed-phase h.p.l.c. This was performed using either 2.1 mm or 1 mm internal diameter columns essentially as described in Hughes *et al.* (1990).

Separation of peptides by SDS/PAGE and electroblotting. Native jacalin (F2 fraction), CNBr and Glu-C peptides derived from electroeluted α- and α'-subunits were electrophoresed on Tris/Tricine gels (Schägger & von Jagow, 1987) and then electroblotted on to PVDF membranes with 20 mM-Tris/HCl/192 mM-glycine, pH 8.3, as a transfer buffer. The membranes were stained with 0.1% Amido Black dissolved in 5% acetic acid/20% methanol and destained with water. Bands of interest were cut out and kept frozen until *N*-terminal sequencing was performed.

Peptide characterization. *N*-Terminal sequence determination was carried out with the models 477A and 473A pulsed liquid-phase microsequencers from Applied Biosystems using standard normal-1 and blott-1 programs.

Sugar determination. This was performed using the Schiff reagent (PAS staining) as described by Kapitany & Zebrowski (1973). Alternatively, the glycan-detection kit from Boehringer was used according to the manufacturer's instructions.

Electron-microscopic visualization of protein. This was done after negative staining following standard protocols (Engel & Furthmayr, 1987) except for some modifications. Grids with thin collodium films were coated with ultrathin carbon films by carbon evaporation, rendered hydrophilic by glow discharge and wetted (45 s) with 10 mM-Tris/HCl/10 mM-NaN_3/1 mM-$MgCl_2$, pH 7.0. The protein (2 μg/ml in 0.2 M-ammonium bicarbonate, pH 7.9) was applied (45 s), washed with water (5 s) and stained twice (5 and 10 s) with freshly prepared uranyl formate.

Sedimentation-velocity and sedimentation-equilibrium studies. These were performed at 20 °C as described by Wright *et al.* (1980) in a Beckman model E analytical ultracentrifuge equipped with absorption optics. The buffer used throughout was 20 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl. The partial specific volume for jacalin was arbitrarily taken as 0.72 cm^3/g, and the density, ρ, = 1.006 g/ml. Values for the weight-average molecular mass were derived from short-column equilibrium centrifugation (Yphantis, 1960).

RESULTS AND DISCUSSION

Jacalin was isolated by DEAE-trisacryl chromatography and yielded three fractions designated F1, F2 and F3 (Aucouturier *et al.*, 1989). Each fraction recovered was further purified by affinity chromatography on a D-melibiose–minileak conjugate. In agreement with Aucouturier *et al.* (1987, 1989), F1 and F2 fractions exhibited two discrete bands on SDS/PAGE with apparent molecular masses of 15 kDa and 12 kDa, in an approximate ratio of 1:3 (as estimated by Coomassie Blue staining). They will be referred to as α'-chain (15 kDa) and α-chain (12 kDa) as already shown by others (Roque-Barreira & Campos-Neto, 1985; Aucouturier *et al.*, 1989). In agreement with Young *et al.* (1989), reversed-phase h.p.l.c. of the jacalin F2 fraction exhibited three well-separated peaks (termed β1, β2, β3 by Young *et al.*, 1989) and a later eluted broad peak that was itself preceded by a shoulder which was shown by SDS/PAGE to contain largely enriched α'-chain. The descending limb of the main peak contained pure α-chain.

Microsequencing of β1, β2 and β3 displayed the same primary structures as those described by Young *et al.* (1989), i.e. polypeptides containing 20 amino acids (molecular mass 2.1 kDa), except for β3 which had asparagine instead of aspartic acid as the *N*-terminal residue. Because the α'-polypeptide could not be fully separated from the α-chain on a C4 reversed-phase column, the F2 jacalin fraction was run on an SDS/polyacrylamide gel and bands were either blotted on to PVDF membranes for *N*-terminal amino acid sequence determination or electroeluted from the gel for chemical and proteolytic cleavage.

The complete amino acid sequences of these major large α- and α'-subunits are summarized in Fig. 1 and comprise 133 amino acids corresponding to a calculated molecular mass of 14.7 kDa (sugar not included). Carboxypeptidase Y digestions of β-subunit-free α- and α'-chain released leucine, serine and tyrosine after 2 h of digestion, data that warrant an unequivocal identical *C*-terminal sequence for both subunits.

Our sequence data clearly show that the minor α'- and the major α-subunits are indistinguishable from each other at the protein level with the exception of a valine-for-isoleucine interchange at position 113. It should be noted that positions 31

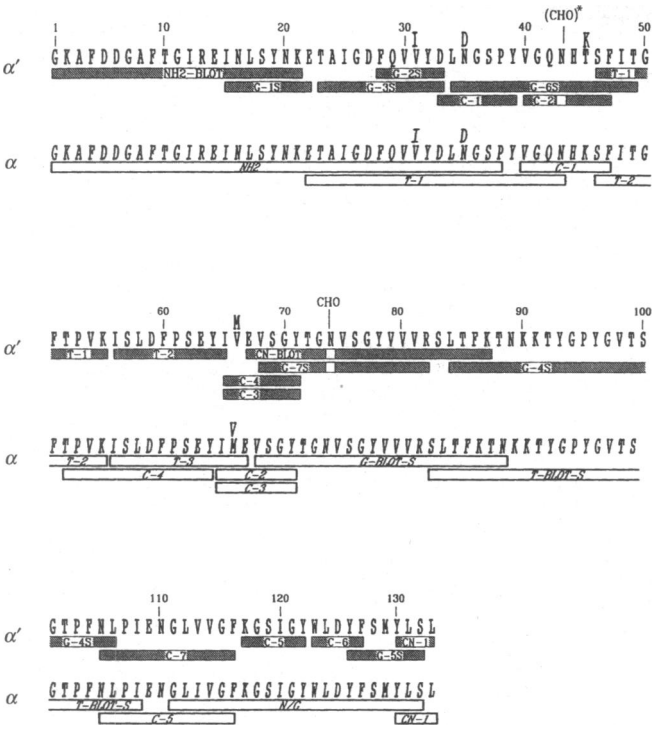

Fig. 1. Amino acid sequences of α′- and α-subunits of jacalin

The one-letter code for amino acids was used. T, C and G: tryptic, chymotryptic and Glu-C peptides; the letter S following the peptide number refers to succinylated material; N/G, peptide derived from cleavage between asparagine and glycine; CN, CNBr peptides; NH_2-BLOT, *N*-terminal sequence of the α′-chain after electrophoresis of native jacalin and transfer on to PVDF membranes; NH_2, *N*-terminal sequence of h.p.l.c.-purified α-chain; small open rectangles indicate that no PTH derivatives were recovered; CHO, asparagine-linked carbohydrate; (CHO)*, asparagine-linked carbohydrate found in the variant peptide C-2 containing threonine at position 45 (see the text). Note that rectangles represent the extent of the sequence determined and not the length of the relevant peptide.

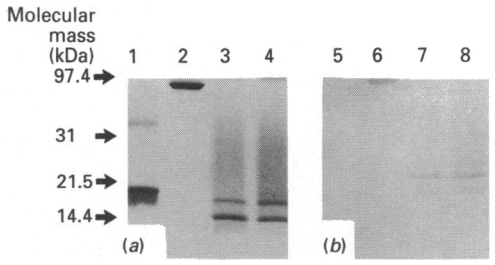

Fig. 2. Sugar detection on SDS/PAGE patterns of native jacalin

(*a*) Control staining by Coomassie Blue: lanes 1, 2, 3 and 4, myoglobin, transferrin, DEAE-trisacryl-purified jacalin and DEAE-cellulose-purified jacalin respectively. Protein concentrations: myoglobin and transferrin, 2.5 μg; jacalin, 10 μg. (*b*) Lanes 5–8, same as in (*a*) but proteins (10 μg in all lanes) were stained with Schiff reagent.

and 66 in α′- and α-chains contain an alternative hydrophobic amino acid (Val/Ile and Met/Val respectively) and that position 45 in the α′-polypeptide has threonine or lysine. Thus the chains exhibit microheterogeneity, a fact that is compatible, at least in part, with the presence of several isolectin forms of jacalin (Vijayakumar & Forrester, 1986; Ahmed & Chatterjee, 1989; Young *et al.*, 1989). Therefore the primary structures determined here are indeed a composite sequence of several distinct polypeptides. The structure is in good agreement with that reported for the α-chain by Young *et al.* (1991), except that they identified other substitutions at positions 45, 67, 72, 74 and 102. Because the origin of the jacalin seeds that they used could not be ascertained, the presence of additional minor genetic variants of the α-chain is not unexpected.

It has been reported that only the α′-subunit was glycosylated, as revealed by the Schiff reagent after SDS/PAGE (Roque-Barreira & Campos-Neto, 1985; Aucouturier *et al.*, 1989). The jacalin fraction F2 was used to demonstrate the presence of sugars, as detected by two distinct methods, (1) metaperiodate oxidation and labelling of the sugar with a steroid, digoxigenin, followed by immunochemical detection on nitrocellulose filters with a specific antibody (Boehringer glycan detection kit), and (2) by using the Schiff reagent. The first method (results not shown) showed that both α′- and α-chains were labelled, in contrast with BSA and myoglobin used as negative controls. Fig. 2 shows the labelling pattern with the Schiff reagent, using transferrin and myoglobin as positive and negative controls respectively. It can be seen that both α′- and α-chains are stained and that α′-chain is more heavily glycosylated (approximate ratio of 2:1) than α-chain, the latter chain accounting for about 75% of the content of this F2 fraction (Aucouturier *et al.*, 1989).

Both chains share three possible *N*-linked glycosylation sites at positions 16, 35 and 74 in the consensus sequence Asn-X-Thr/Ser (Marshall, 1972). A fourth possible site was found at position 43 in the α′-chain. At position 16, asparagine was detected in good yield as a phenylthiohydantoin (PTH) derivative, indicating that this residue is not glycosylated. At position 35, asparagine is the major residue, although aspartate was found as an alternative amino acid; this may, however, be due to deamidation during the isolation and cleavage procedures. The major carbohydrate in jacalin is *N*-linked at position 74 in the α′-chain. At this position, no residue could be identified in the high-molecular-mass α′-chain (reported as a blank in CN-BLOT and G-7S, Fig. 1); however, in the α-chain, normal recoveries of PTH-asparagine for Glu-C, tryptic and chymotryptic peptides spanning this region were obtained. Asparagine (position 43) was not detected in the sequence of the α′ chymotryptic peptide C-2 (Fig. 1) which contains a consensus sequence for *N*-linked glycosylation (threonine at position 45). In contrast, a normal yield of PTH-asparagine was obtained for the Glu-C peptide, G-6S, which contains lysine at position 45. This suggests that the α′-polypeptide is partially glycosylated at position 43.

Our finding is consistent with the data of Capon *et al.* (1990), who isolated a single Pronase glycopeptide from jacalin and showed that an asparagine residue was linked to a biantennary neutral oligomannoside of molecular mass 1272 Da. From microsequencing data only, no firm evidence for *N*-linked glycosylation was found in the α-chain. This is in contrast with the positive detection of sugars by two independent methods which both revealed that the two chains were labelled. It is highly likely that a minor population of glycosylated α-chain variants escaped detection during microsequencing.

The jacalin F2 fraction was examined under the electron microscope and the electron micrograph is shown in Fig. 3. Particles were well resolved by the negative staining technique and they appeared as structures composed of four symmetrical globuli per molecule (indicated by arrows); at positions of the grid at which the stain was thin, individual ellipsoid subunits could sometimes be distinguished. As the limit of resolution of the method is about 12–15 kDa (Engel & Furthmayr, 1987), the electron micrograph of jacalin suggests that this lectin is in the form of a symmetrical tetramer in solution. Because the difference in mass between the α- and α′-chains cannot be distinguished, the relative proportion of the two in the tetrameric structure cannot be assessed.

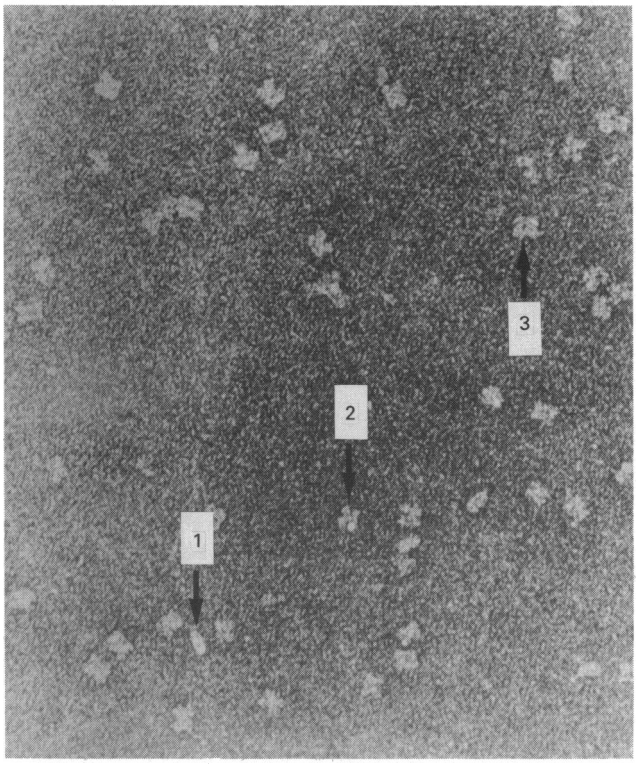

Fig. 3. Electron micrograph of native jacalin

The final magnification was 500000-fold (see the Materials and methods section). 1, side view; 2, ellipsoid form; 3, globular form.

Sedimentation-equilibrium data indicated an average molecular mass of 64 kDa for jacalin in phosphate-buffered saline, pH 7.3, and sedimentation-velocity studies allowed us to calculate an $s_{20,w}$ value of 4.4, corresponding to a molecular mass of 65 kDa. This value rules out all previously postulated molecular-mass values (Roque-Barreira & Campos-Neto, 1985; Appukutan & Basu, 1985; Young *et al.*, 1989; Lee *et al.*, 1989). In addition to the presence of α- and α'-subunits, jacalin contains three distinct forms of a small 20-residue peptide, referred to as β-chain (Young *et al.*, 1989). These data are entirely consistent with a α/α'-tetramer containing two to four β-chains.

On the basis of these results, the number of binding sites per $\alpha(\alpha')$-protomer should be recalculated; Appukuttan & Basu (1985) and Sastry *et al.* (1986) determined two binding sites per assumed tetrameric 39.5 kDa jacalin. Taking the experimentally determined molecular mass value of 65 kDa, the number of binding sites per jacalin molecule becomes 3.3, i.e. approx. 0.8 sites on each $\alpha(\alpha')$-protomer. This value appears to be more realistic than the unexpected 'half of the sites' reactivity, initially reported also for two other lectins (Lotan *et al.*, 1974; Bessler & Goldstein, 1974); in both cases, results were corrected to suggest one full binding site on each of the four subunits (De Boeck *et al.*, 1984; Roberts & Goldstein, 1984).

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