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The *fuc1* gene product (20 kDa FUC1) of *Pisum sativum* has no α-L-fucosidase activity

Teresa Tarragó[1], Immaculada Martínez[1], Margarita Torrent[1], Anna Codina[2], Ernest Giralt[2], Pere Puigdomènech[1] and Dolores Ludevid[1,*]

[1]*Departament de Genètica Molecular, Institut de Biologia Molecular de Barcelona, (CSIC), 18-26 Jordi Girona, 08034 Barcelona, Spain (*author for correspondence; e-mail dlmgmd@cid.csic.es);* [2]*Departament de Química Orgànica, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain*

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Abstract

An α-L-fucosidase purified from pea (*Pisum sativum* L. cv Alaska) epicotyl was previously described as a cell wall enzyme of 20 kDa that hydrolyses terminal α-L-fucosidic linkages from oligosaccharide fragments of xyloglucan. cDNA and genomic copies were further isolated and sequenced. The predicted product of the cDNA and the genomic clone (*fuc1*), was a 20 kDa protein containing a signal peptide and five cysteines. This was the first α-L-fucosidase gene to be cloned in plants but its fucosidase activity has not been demonstrated. Here, our biochemical and immuno analyses suggest that *fuc1* does not encode an α-L-fucosidase. Pea *fuc1* expressed in *Escherichia coli*, insect cells and *Arabidopsis thaliana* produced recombinant proteins without α-L-fucosidase activity. Pea plants had endogenous α-L-fucosidase activity, but the enzyme was not recognised by an antibody produced against recombinant FUC1 protein expressed in *E. coli*. In contrast, the antibody immunoprecipitated a 20 kDa protein which was inactive. By chromatographic analysis of pea protein extracts, we separated α-L-fucosidase-active fractions from the 20 kDa protein fractions. We conclude that the α-L-fucosidase activity is not attributable to the 20 kDa FUC1 protein. A new function for *fuc1* gene product, now named PIP20 (for protease inhibitor from pea) is proposed.

Abbreviations: AMC, 7-amino-methyl-coumarin; ArFUC1, *A. thaliana* recombinant protein; BSA, bovine serum albumin; DTT, dithiothreitol; ErFUC1, *E. coli* recombinant protein; 2FL, 2′-Fucosyl-lactose; 2FL-AMC, 2′-fucosyl-lactose 7-amino-methyl-coumarin; L, lactose; L-AMC, lactose 7-amino-methyl-coumarin; 2-ME, 2-mercaptoethanol; ORF, open reading frame; α-pFuc, polyclonal antibody against purified pea α-L-fucosidase; PMSF, phenylmethyl-sulfonil fluoride; α-rP20, polyclonal antibody against ErFUC1; Sf9, *Spodoptera frugiperda* 9; SrFUC1, Sf9 recombinant protein; TEA, triethanolamine; TLC, thin-layer chromatography

Introduction

α-L-fucosidases hydrolyse α-L-fucosyl residues. They have been isolated from man (Occhiodoro *et al.,* 1989), fungi (Bahl, 1970), bacteria (Wong-Madden and Landry, 1995) and plants. At present, there are only three reports of fucosidase purification in plants; from almond seeds (Ogata-Arakawa *et al.,* 1977), from pea epicotyls (Augur *et al.,* 1993) and recently from *Brassica oleracea* (de la Torre *et al.*, 2002). A purified pea α-L-fucosidase has been described as a cell-wall protein of 20 kDa which hydrolyses oligosaccharide substrates containing fucosyl-α-1,2-Gal linkages (Augur *et al.*, 1993). The amino acid sequences of the N-terminal region and one internal peptide allowed Augur *et al.* (1995) to design primers and isolate the genomic and cDNA clones encoding the pea α-L-fucosidase. The α-L-fucosidase gene *fuc1* (accession number X82595) was mainly expressed in the meristematic and elongating tissues of

pea plants. The 217 amino acid protein showed no sequence homology to other fucosidases, but it had an N-terminal region with 43–33% identity to two Kunitz-type trypsin inhibitors. The *fuc1* sequence was the first fucosidase gene cloned in plants. In this report, we demonstrate that the *fuc1* gene product has no α-L-fucosidase activity. According to our results, the α-L-fucosidase activity was not attributable to the 20 kDa protein previously described (Augur *et al.*, 1993). This conclusion is supported by activity assays on *fuc1* gene expressed in heterologous systems, immunoprecipitation experiments and chromatographic analysis of pea soluble proteins.

Materials and methods

Plasmid constructs

The *fuc1* gene (Augur *et al.*, 1995) full-length ORF (open reading frame) sequence was obtained by PCR with the oligonucleotides F1 (CGGGATCCATATGAAACCTCTTTCAC) and F2 (GCGGATCCTCAAACAACAGACTTAA) and the gene *fuc1* sequence (accession number X82595; Augur *et al.*, 1995) as template. PCR conditions were as follows: 92 °C for 2 min, then 30 cycles of 92 °C for 40 s, 40 °C for 40 s and 72 °C for 90 s, followed by 72 °C for 5 min. The PCR product was confirmed by DNA sequencing and was introduced into the *Eco*RV site of plasmid pKS (Stratagene) to generate the plasmid pKSFUC1. To express FUC1 in *Escherichia coli*, the *fuc1* coding sequence without the signal peptide (amino acids 27–217) was created by PCR amplification over pKSFUC1 with the primers F3 (TTCGAAGCTTCATATGGAAGATGTTGAGCAAGTA) and F2. PCR conditions were as described above. The PCR fragment was inserted in *Nde*I-*Bam*HI sites of pET14b (Novagen) to obtain pET14bFUC1. pFastBac1FUC1 used in insect cell transfection was obtained by inserting the *fuc1* coding region (*Sal*I-*Xba*I fragment) from pKSFUC1 into pFastBac1 vector (Gibco-BRL). For plant transformation, the binary plasmid pBin19FUC1 was obtained by inserting the full-length *fuc1* ORF from pKSFUC1 digested with *Kpn*I and *Xba*I into a pBin19 vector (Bevan, 1984) under the cauliflower mosaic virus 35S promoter and the *ocs* polyadenylation regulatory sequence. PCR products and constructs were confirmed by DNA sequencing.

fuc1 *gene expression in* E. coli

The recombinant plasmid pET14bFUC1, which directs synthesis of the FUC1 protein (amino acid residues 27–217) fused to a 6× His-tag at its N-terminus, was introduced into BL21 cells. The *E. coli* recombinant protein (ErFUC1) was purified from inclusion bodies by Ni^{2+} affinity chromatography in denaturing conditions in buffer D (20 mM Tris-HCl pH 7.9, 6 M urea, 0.5 M NaCl). For the renaturation process, the ErFUC1 level was adjusted to 50 ng/μl in buffer D containing 20 mM 2-mercaptoethanol (2-ME) and dialysed progressively to remove first the urea and then the 2-ME. The final oxidation of cysteines was carried out by slow stirring for 12 h at room temperature. Folding was tested by disulfide-bond formation and resistance to trypsin digestion as described (Codina *et al.*, 2001).

fuc1 *gene expression in insect cells*

Sf9 cells were transfected with FUC1 baculovirus generated from the pFastBac1FUC1 by using the Bac-to-Bac Baculovirus Expression System (Gibco-BRL). Cells and media from transfected and non-transfected cells were collected after 72 h of culture for protein expression analyses and measurement of α-L-fucosidase activity. Cells were sonicated in buffer B (100 mM sodium acetate pH 6.0) to obtain a cell lysate and cleared by centrifugation. The corresponding growth media were concentrated in buffer B with Biomax-5 filters (Millipore, nominal molecular mass limit 5 kDa). α-L-Fucosidase activity was analysed in cell lysates and concentrated media. Proteins were determined by the Bradford assay (BioRad). The insect recombinant protein, SrFUC1, was analysed by SDS-PAGE and immunoblot.

fuc1 *gene expression in plants*

To generate transgenic *A. thaliana* plants expressing *fuc1* gene, the binary vector pBin19FUC1 was introduced into *A. thaliana* plants (ecotype RLD) via *Agrobacterium tumefaciens* (Clough and Bent, 1998). A total of 25 kanamycin-resistant transgenic plants were selected and plants with the highest recombinant protein (ArFUC1) levels in the T_1 generation were chosen to obtain the T_3 generation. Total proteins were extracted in a buffer containing 50 mM Tris-HCl pH 8, 50 mM NaCl, 1% SDS, 5% 2-ME and protease inhibitors (10 μg/ml aprotinin, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1 μg/ml E64,

0.1 mM phenylmethylsulfonile fluoride, PMSF). Protein extracts were quantified by the Bradford assay and analysed by SDS-PAGE and immunoblot. For α-L-fucosidase activity assays, recombinant protein ArFUC1-enriched extracts were obtained from leaves of *A. thaliana* seedlings (20 days old) as described below for pea leaves.

Pea FUC1 protein purification

Pea FUC1-enriched extracts were obtained as follows. Young leaves from ten 20-day old pea plants (*Pisum sativum* L. cv. Alaska) were homogenized in an ice-cold mortar with 20 ml of ice-chilled 100 mM sodium acetate pH 6.0, containing 1 mM dithiothreitol (DTT) and protease inhibitors. After sonication the suspension was centrifuged at 15 000 $\times$ *g* for 30 min at 4 °C and the supernatant was precipitated with 43% ammonium sulfate for 30 min on ice and then centrifuged at 8000 $\times$ *g* for 30 min at 4 °C. The precipitate was suspended in 10 ml of 50 mM sodium citrate pH 6.0, dialysed against the same buffer and centrifuged at 14 000 $\times$ *g* for 30 min. Aliquots (1 ml) of the supernatant were lyophilised and stored at -80 °C until use.

Pea FUC1-enriched extract was applied to a NAP 5 column (Pharmacia Amersham), eluted in 1 ml of buffer C (20 mM Tris-HCl pH 7.0) and then loaded onto an anion exchange column (HiTrapQ, Pharmacia Amersham) equilibrated with the same buffer. The column was washed with buffer C, followed by a linear gradient of NaCl (0–300 mM) in the same buffer (flow rate 0.5 ml/min) and a plateau of 250 mM NaCl as shown in Figure 3A. Fractions of 0.5 ml were collected and 18 μl of each fraction was analysed by SDS-PAGE and immunoblot. When indicated, chromatographic fractions were five-fold concentrated before to be electrophoresed.

Antibodies and immunological methods

The polyclonal antibodies α-rP20 were raised in rabbits injected with purified ErFUC1. The cross-reactivity of this antibody was tested for lysates from transfected Sf9 cells, transgenic *A. thaliana* plants and pea protein extracts. The anti α-fucosidase antibody, α-pFuc, raised against purified pea α-L-fucosidase (Augur *et al.*, 1993) was kindly gifted by Ch. Augur. For immunoblots, nitrocellulose sheets were incubated with α-rP20 antiserum (1:3000 dilution) or with α-pFuc antiserum (1:5000 dilution) for 1 h at room temperature and immunoreactive bands were detected by the ECL system (Pharmacia Amersham). The amount of pea FUC1 and recombinant FUC1 proteins present in the extracts was measured by ELISA with α-rP20 (1:3000 dilution) and anti-rabbit peroxidase conjugate (1:8000 dilution) (Sigma). ErFUC1 protein was used as standard.

Pea FUC1 protein was purified from pea extracts by immunoaffinity with anti-rabbit M-280 Dynal beads. Beads were coated either with α-rP20 antibody or α-pFuc antibody in PBS and 0.1% bovine serum albumin (BSA). After several washes the antibodies were cross-linked to the anti-rabbit IgG from the beads with 20 mM dimethylpimelidate (Sigma) in 200 mM triethanolamine (TEA) pH 9 for 1 h. Beads were incubated for 2 h with 200 mM TEA and washed in PBS-0.1% BSA. Pea extracts were incubated first with uncoated beads (pre-clearing) and then, with immobilised antibodies. The immunopurified FUC1 protein was eluted from beads and analysed by SDS-PAGE and immunoblots. The α-L-fucosidase activity present in the immunopurified FUC1 protein was also determined. Non-immune serum was used as control.

α-L-fucosidase activity assay

2′-Fucosyl-lactose 7-amino-methyl-coumarin (2FL-AMC, New England Biolabs) was used as substrate (Prakash and Vijay, 1983). 2FL-AMC (1 nmol) was incubated for 12 h at 37 °C with various protein extracts in a 10 μl reaction mix containing 50 mM sodium citrate pH 6.0. The AMC-labelled oligosaccharide was extracted with 10 μl of butanol and analysed by thin-layer chromatography (TLC, Whatman) as described (Ogata-Arakawa *et al.*, 1977). Controls included the disaccharide product lactose 7-amino-methyl-coumarin (L-AMC) resulting from the digestion of 2FL-AMC with 1 Unit of *Xanthomonas manihotis* α-(1,2)-fucosidase (New England Biolabs) and undigested 2FL-AMC. Substrate and reaction product were illuminated by a 312 nm UV lamp and quantified in a Fluor-S Multi Image device with Quantity One software (BioRad). The ErFUC1, SrFUC1, ArFUC1 and pea FUC1 proteins were quantified from extracts by ELISA. Before enzymatic assays, ErFUC1 protein solution and anion exchange chromatography fractions were desalted. Chromatographic fractions were concentrated 5-fold in 50 mM sodium citrate pH 6.0.

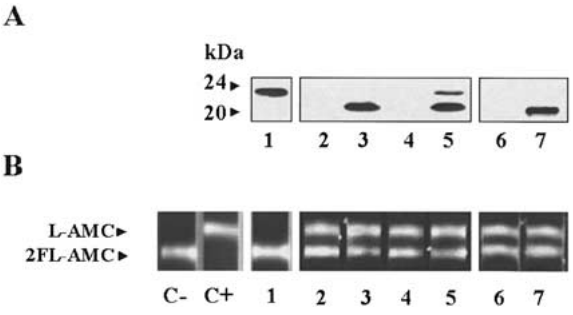

Figure 1. The *fuc1* gene product is not active when expressed in heterologous systems. A. Immunoblot analysis of *FUC1* recombinant proteins by using the α-rP20 antibody raised against the recombinant pea FUC1 protein produced in *E. coli* (ErFUC1). A 20 ng portion of ErFUC1 (lane 1), extracellular media of Sf9 untransformed cells (lane 2) and transformed cells (lane 3), cell protein extracts of Sf9 untransformed cells (lane 4) and transformed cells (50 ng of recombinant pea FUC1 protein produced in insect cells, SrFUC1, lane 5), 5 μg of total soluble proteins of *Arabidopsis* wild-type plants (lane 6) and 20 ng of recombinant pea FUC1 protein from transformed *A. thaliana* plants (ArFUC1, lane 7) were loaded. Molecular mass is indicated on the left. B. α-L-fucosidase activity assay. Lane 1, activity of 200 ng of ErFUC1. Lanes 2 and 3, growing media of untransformed (lane 2) and *fuc1*-transformed Sf9 cells (lane 3). Lanes 4 and 5, cell extracts from non-transformed (lane 4) and *fuc1* transformed Sf9 cells (150 ng SrFUC1, lane 5). Lane 6, protein extracts of wild-type *A. thaliana* plants. Lane 7, protein extracts of transgenic *A. thaliana* plants over-expressing the *fuc1* gene (70 ng ArFUC1). Lane C−, undigested substrate 2FL-AMC used as negative control. Lane C+, the reaction product L-AMC obtained after digestion of the 2FL-AMC substrate with 1 unit of commercial α-L-fucosidase.

Results

α-L-Fucosidase activity assay of fuc1 *gene product (20 kDa FUC1) expressed in* E. coli*, insect cells and* Arabidopsis

To examine the α-L-fucosidase activity of the *fuc1* gene product, *E. coli*, insect cells and *A. thaliana* plants were used as expression systems. ErFUC1 was used both for antibody production (α-rP20) and for α-L-fucosidase activity assays. The expression products of the *fuc1* gene in *E. coli*, insect cells and plants were analysed by immunoblot using the polyclonal antibody α-rP20 (Figure 1A). ErFUC1 was expressed in *E. coli* as a fusion protein containing a His-tag sequence. Consequently, the apparent molecular weight of ErFUC1 (Figure 1A, lane 1) was higher than those corresponding to SrFUC1 (Figure 1A, lane 3) and ArFUC1 (Figure 1A, lane 7). To determine the α-L-fucosidase activity of the recombinant proteins we used an enzymatic assay based in the hydrolysis of 2′-fucosyl-lactose linked to coumarin (2FL-AMC) (Prakash and Vijay, 1983) (Figure 1B). The product of the enzymatic hydrolysis of fucose from the substrate (2FL-AMC) was analysed by TLC. The high-mobility band in the positive control (Figure 1B, C+) corresponds to the disaccharide product and the low mobility band in the negative control (Figure 1B, C−) corresponds to the substrate. ErFUC1 expressed in *E. coli* and purified by affinity (Figure 1A, lane 1) was inactive even when 200 ng of recombinant protein was used (Figure 1B, lane 1). It could be that the protein expressed in *E. coli* was not properly folded or that some post-translational modification on the protein would be essential for the activity. To explore this hypothesis, we expressed the *fuc1* gene in eukaryotic systems such as insect cells and *A. thaliana*. When the complete *fuc1* coding region was inserted into a baculovirus vector to transform Sf9 insect cells, the resultant 20 kDa SrFUC1 protein was present in cell extracts and partially secreted to the extracellular medium (Figure 1A, lanes 5 and 3, respectively). The immunoreactive band of 23 kDa which was detected only in transformed cell extracts (Figure 1A, lane 5) correspond to unprocessed protein. The α-L-fucosidase activity was analysed in both extracellular media and protein extracts of Sf9 cells transformed with *fuc1* (Figure 1B, lanes 3 and 5). Media and protein extracts of non-transformed cells were always used as controls (Figure 1A, lanes 2 and 4, respectively). Since endogenous α-L-fucosidase activity was detected in non-transformed Sf9 cells (Figure 1B, lanes 2 and 4), we expected a significant increase in α-L-fucosidase activity in transformed cells. Transformation with *fuc1* had no effect on α-L-fucosidase activity (Figure 1B, lanes 3 and 5). These results indicate that plant-specific cofactors or post-translational modifications may be responsible for this enzymatic activity. Therefore, transgenic *A. thaliana* plants over-expressing ArFUC1 protein (Figure 1A, lane 7) were produced. In transgenic *A. thaliana* plants the ArFUC1 protein was also inactive (Figure 1B, lane 7). Wild-type *A. thaliana* plants had endogenous α-L-fucosidase activity (Figure 1B, lane 6), but the presence of *fuc1* gene product in the transgenic lines did not increase enzyme activity (Figure 1B, compare lanes 6 and 7). This result strongly suggests that endogenous *A. thaliana* α-L-fucosidase activity results from proteins unrelated to ArFUC1 (Figure 1A, lane 6).

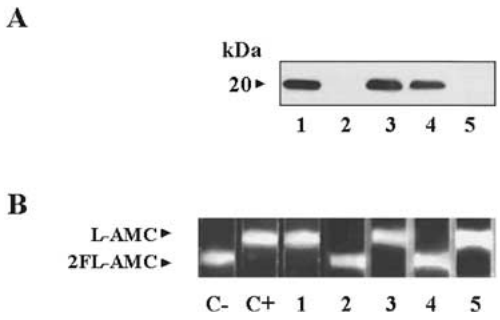

Figure 2. Antibodies α-rP20 do not recognize pea α-L-fucosidase activity. A. Characterization of pea immunoprecipitated proteins by immunoblot with antibodies α-rP20 raised against the recombinant ErFUC1 protein produced in *E. coli*. Lane 1, pea protein extracts. Lane 2, protein immunoprecipitated with pre-immune serum used as control. Lane 3, control wash fraction corresponding to pre-immune serum. Lane 4, proteins immunoprecipitated with α-rP20 magnetic beads. Lane 5, wash fraction from α-rP20 magnetic beads. Molecular mass is indicated on the left. B. α-L-Fucosidase activity of α-rP20 immunoprecipitated pea extracts. Lanes 1–5 show the activity of the corresponding samples used in the immunoblot assay depicted in A. Lanes C− and C+ as in Figure 1.

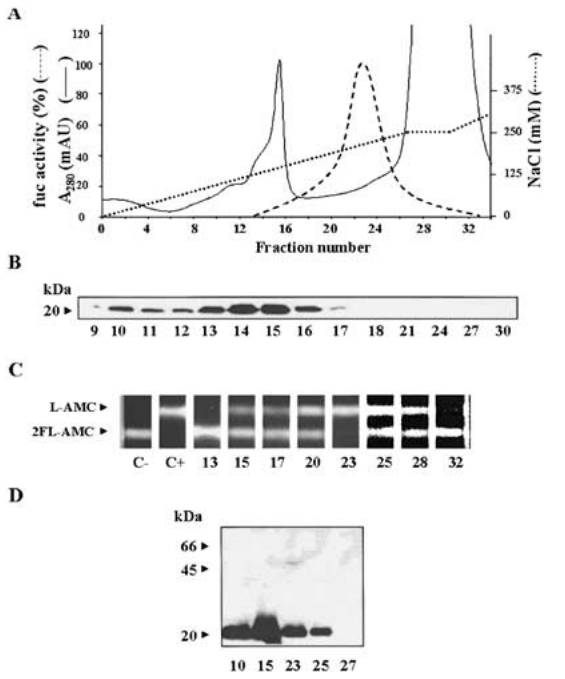

Figure 3. Anion exchange chromatography of pea extracts. A. Representative elution profile of pea extracts separated by anion exchange chromatography. α-L-fucosidase activity profile from the TLC assay shown in C is also indicated on the graphic. B. Western blot with the α-rP20 antibody (raised against the recombinant ErFUC1 protein produced in *E. coli*) showing the presence of the FUC1 protein in the column fractions. Of each fraction 18 μl was loaded per lane. Molecular mass is indicated on the left. C. α-L-Fucosidase activity assay of the indicated column fractions. Lanes C− and C+ as in Figure 1. D. Western blot analysis of 5-fold concentrated chromatographic fractions with the α-pFuc antibody raised against purified pea FUC1 protein (Augur *et al.*, 1995). Molecular masses are indicated on the left.

Antibodies against ErFUC1 protein do not recognise the pea α-L-fucosidase activity

Since the recombinant *fuc1* gene product did not exhibit α-L-fucosidase activity when expressed in heterologous systems, the next question was whether the endogenous pea *fuc1* product (20 kDa FUC1) had α-L-fucosidase activity. To explore the activity of 20 kDa FUC1, we isolated it from pea soluble protein extracts by immunoaffinity on magnetic beads coated with α-rP20 antibody. As shown in the Figure 2A, the 20 kDa FUC1 protein present in pea extracts (lane 1) was recognized by the antibody and eluted from the α-rP20 magnetic beads (lane 4). No traces of 20 kDa FUC1 were observed in washes previous to elution (Figure 2A, lane 5). As expected, the pre-immune serum cross-linked to magnetic beads did not recognize the 20 kDa FUC1 protein of pea extracts (Figure 2A, lane 2) which was recovered in the wash fraction (Figure 2A, lane 3). In fucosidase activity assays of the different fractions, we found that the immunopurified pea 20 kDa FUC1 did not cleave 2FL-AMC (Figure 2B, lane 4), but the wash fraction exhibited α-L-fucosidase activity (Figure 2B, lane 5). Moreover, in preimmune beads experiment, the α-L-fucosidase activity was also detected in the wash fraction (Figure 2B, lane 3). These results already suggested that the α-L-fucosidase activity present in pea extracts was not recognised by α-rP20 antibody and that likely, the 20 kDa FUC1 protein was unrelated to α-L-fucosidase activity. Then, we examined whether pea 20 kDa FUC1 and pea α-L-fucosidase enzyme were two different proteins by a biochemical approach.

α-L-Fucosidase activity is resolved from fuc1 *gene product (20 kDa FUC1) by anion exchange chromatography*

Augur and co-workers have purified the pea α-L-fucosidase by selective extraction from pea epycotyls with a combination of CM-Sepharose and preparative isoelectric focusing (Augur *et al.*, 1993). On CM-Sepharose, the pea α-L-fucosidase activity was eluted at 150–200 mM NaCl and SDS-PAGE analysis and silver staining of these fractions revealed a 20 kDa protein that co-purified with α-L-fucosidase activity on isoelectric focusing. Here, by introducing several

changes in the purification protocol, we were able to separate α-L-fucosidase activity from the 20 kDa *fuc1* gene product. Young leaves from 20-day old pea plants were collected and used as starting plant material. Total soluble proteins were extracted and precipitated with 45% ammonium sulfate and loaded on an anion exchange chromatography column (HiTrap Q) (Figure 3). The chromatographic pattern showed a small sharp peak at 120 mM NaCl and a broad peak at 250 mM NaCl (Figure 3A). Immunoblot analysis with α-rP20 antibody of all fractions collected (Figure 3B) indicated that the 20 kDa FUC1 protein eluted at the first peak reaching a maximum between fractions 14 and 15. Nevertheless, fucosidase activity did not co-elute with 20 kDa FUC1 protein, and the pattern of active fractions was shifted from the 20 kDa peak to higher NaCl concentrations (200 mM NaCl) (Figure 3B, see fraction 23). Additionally, fractions enriched in α-L-fucosidase activity (Figure 3C, fraction 23) and in 20 kDa FUC1 protein (Figure 3B, fraction 15) were concentrated and analysed by immunoblot (Figure 3D) with α-pFuc antiserum raised against the pea 20 kDa FUC1 purified protein (Augur *et al.*, 1993). Interestingly, a faint band of 55 kDa was recognized by α-pFuc antiserum (Figure 3D, fraction 23), while that band was not recognized by α-rP20 antibodies (not shown). As expected, one band of 20 kDa corresponding to the pea FUC1 protein was clearly enriched in fraction 15 and significant amounts of this protein were also detected in fractions 10, 23 and 25 as consequence of the concentration of the corresponding samples. Since the antibody α-pFuc recognized two bands (55 kDa and 20 kDa), the next question was if this antibody was capable of immunoprecipitate α-L-fucosidase activity from pea extracts. Then, pea extracts were immunoprecipitated by using magnetic beads coated with α-pFuc antiserum as described. As can be seen in Figure 4, α-pFuc antiserum immunoprecipitated α-L-fucosidase activity from pea extracts (lane 3), a result that is in contrast with that obtained when using α-rP20 antibody (see Figure 2). The fact that both α-rP20 and α-pFuc antibodies recognized the 20 kDa FUC1 protein but only the α-pFuc antibody immunoprecipitated α-L-fucosidase activity strongly suggest that α-L-fucosidase activity is not associated with the 20 kDa protein.

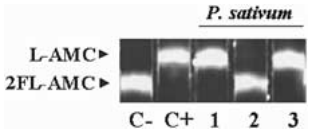

Figure 4. Antibodies α-pFuc recognize pea α-L-fucosidase activity. α-L-fucosidase activity assay. Lane 1, non-immunoprecipitated pea extracts. Lane 2, immunoprecipitated extracts with non-immune serum. Lane 3, immunoprecipitated extracts with the α-pFuc serum raised against purified pea FUC1 protein (Augur *et al.*, 1995). Lanes C− and C+ as in Figure 1.

Discussion

α-L-fucosidase activity is reported in pea epicotyl extracts (Farkas *et al.*, 1991), almond seeds (Ogata-Arakawa *et al.*, 1977), *Brassica* extracts (de la Torre *et al.*, 2002) and *Rubus fructicosus* L. cell suspensions (Vargas-Rechia *et al.*, 1998). Previous reports have claimed that in pea α-L-fucosidase is a cell wall protein of 20 kDa encoded by the *fuc1* gene (Augur *et al.*, 1995). Here, by using protein extracts from pea leaves and *A. thaliana*, we show the presence of such activity in these plants. However, this fucosidase activity was not related to the *fuc1* gene product either in pea or in *A. thaliana fuc1* transgenic plants. The *fuc1* coding sequence, when expressed in *E. coli*, insect cells and *A. thaliana*, produced FUC1 recombinant proteins which were inactive and did not hydrolyse the terminal fucosyl residue from fucosyl-lactose. The recombinant proteins had the expected characteristics of the *fuc1* gene product: (1) SrFUC1 and ArFUC1 had a molecular mass of 20 kDa and the His-tagged ErFUC1 had an apparent molecular mass of 23 kDa, and (2) all of them were recognized by the α-rP20 antibody (Figure 1) and α-pFuc antibody (not shown). The absence of α-L-fucosidase activity of *fuc1* gene products expressed in heterologous systems indicated that the FUC1 protein, if active, may be active only in pea plants. In pea plants, however, we observed that the 20 kDa FUC1 protein and the α-L-fucosidase enzyme were two different proteins which we were able to separate by anion exchange chromatography (Figure 3). The α-rP20 antibody recognised the 20 kDa FUC1 protein but not the α-L-fucosidase activity-associated polypeptide. Overall, our findings suggest that *fuc1* does not encode a pea α-L-fucosidase. This result contrasts to those proposed by Augur *et al.* (1995). One possible explanation could be that when α-L-fucosidase protein was purified from pea epycotil extracts in basis of enzymatic activity, it was co- purified with the pea 20 kDa FUC1 protein. If this is the case, the antibody

α-pFuc raised against pea purified α-L-fucosidase (Augur *et al.*, 1993) should recognize both proteins, the 20 kDa FUC1 and the fucosidase enzyme. In fact, when we used an α-pFuc antibody to characterize anion exchange chromatographic fractions, we observed that this antibody recognized the 20 kDa protein but also cross-reacted with a 55 kDa protein present in α-L-fucosidase-active fractions (Figure 3D). Consistent with this hypothesis, the α-pFuc antibody immunopurified the 20 kDa FUC1 protein together with α-L-fucosidase activity (Figure 4) in pea extracts. From these results, it seems that the α-L-fucosidase activity in pea is related with a low abundant 55 kDa polypeptide with some physical and chemical properties very close to those of 20 kDa FUC1 protein. This observation is consistent with the results of de la Torre *et al.* (2002). They isolated two *Arabidopsis* genes, *AtFXG1* and *AtFUC1* (accession numbers AC008113 and AC005851, respectively), which showed α-L-fucosidase activity when expressed in *Pichia pastoris*. *AtFXG1* encodes an α-L-fucosidase (38 kDa) that is active against the XXFG oligosaccharides from xyloglucan and 2′-fucosyl-lactitol, and *AtFUC1*-coded protein (54 kDa) was active only against 2′-fucosyl-lactitol, when expressed in *P. pastoris* cells. These two fucosidase genes did not share any homology with pea *fuc1* gene at either DNA or protein level.

If the 20 kDa FUC1 protein was not a fucosidase, its function is unknown. Indeed, the absence of sequence homology with other fucosidases was described by Augur *et al.* (1995) who found that the N-terminal region of FUC1 protein had 33–43% identity to the N-terminal region of two Kunitz-type trypsin inhibitors. Recently, Codina *et al.* (2002) reported the secondary structure of the ErFUC1 protein. Their data revealed that ErFUC1 is mostly β-protein containing at least twelve, probably anti-parallel, β-strands. Interestingly, this structure has been reported for other proteins belonging to the Kunitz-type trypsin inhibitors family (Blow *et al.*, 1974; Onesti *et al.*, 1991; Dattagupta *et al.*, 1999). Therefore, *fuc1*, as described for related genes coding for protease inhibitors (Koiwa *et al.*, 1997), could be involved in plant defence responses. Recently, a *Glycine max* trypsin inhibitor (accession number AAF87095) with 66% identity to pea FUC1 protein as well as two *Canavalia lineata* Kunitz-type subtilisin inhibitors CLSI-II and CLSI-III (accession numbers JX0311 and JX0310, respectively) (Terada *et al.*, 1994), both with 57% identity to pea FUC1 protein, are annotated in the NCBI Protein Database. Moreover, two protein sequences from *Cicer arietinum* (accession numbers CAB76907 and CAB76906) and ESTs from *Medicago truncatula* (AW126318) and *Lotus japonicus* (AV420785) have recently been identified with a strong homology to *fuc1* gene. According to these data, *fuc1* may encode a protease inhibitor that is specific to leguminous plants. Overall, *fuc1* and its homologues in *Glycine*, *Canavalia, Cicer*, *Medicago* and *Lotus* would form part of the Kunitz-type soybean trypsin inhibitor family. Preliminary results obtained in our laboratory by using *fuc1* transgenic plants point this way since these plants show enhanced resistance to different plant pathogens (results not shown). Finally, in order to clarify the relationship between gene and protein function we propose to rename the *fuc1* gene and the corresponding FUC1 protein as *PIP20* and PIP20 (for protease inhibitor from pea).

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