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A Cherry Protein and Its Gene, Abundantly Expressed in Ripening Fruit, Have Been Identified as Thaumatin-Like[1]

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A 29-kD polypeptide is the most abundant soluble protein in ripe cherry fruit (*Prunus avium* L); accumulation begins at the onset of ripening as the fruit turns from yellow to red. This protein was extracted from ripe cherries and purified by size-exclusion and ion-exchange chromatography. Antibodies to the purified protein were used to screen a cDNA library from ripe cherries. Numerous recombinant plaques reacted positively with the antibodies; the DNA sequence of representative clones encoded a polypeptide of 245 amino acid residues. A signal peptide was indicated, and the predicted mature protein corresponded to the purified protein in size (23.3 kD, by mass spectrometry) and isoelectric point (4.2). A search of known protein sequences revealed a strong similarity between this polypeptide and the thaumatin family of pathogenesis-related proteins. The cherry thaumatin-like protein does not have a sweet taste, and no antifungal activity was seen in preliminary assays. Expression of the protein appears to be regulated at the gene level, with mRNA levels at their highest in the ripe fruit.

Ripening of cherry fruit occurs only during maturation on the tree and is nonclimacteric, i.e. without the autocatalytic burst of ethylene that is characteristic of ripening in tomato, peach, apple, etc. (Hartmann et al., 1987). Like climacteric fruit, the significant changes in structural and biochemical constituents of the cherry fruit (e.g. changes in fruit size, color, sugar content, texture, etc.) are correlated with changes in mRNA and proteins (Fils-Lycaon et al., 1988a, 1988b). Two major components of the protein profile of ripe cherries have been previously described (Krishnan and Pueppke, 1990a, 1990b). The first was a 63-kD soluble invertase thought to be involved in sugar accumulation. The second was a 29-kD polypeptide comprising up to 42% of the total soluble proteins of cherry mesocarp at the ripe stage. This protein was shown to have antigenic homology to a deglycosylated carrot β-fructosidase, although functional activity was not demonstrated. In a study of gene products involved in cherry ripening, we have addressed the question of the identity of this protein. Here we report the purification of the 29-kD polypeptide from ripe cherry, its relation to the previously published protein, the cloning of the corresponding cDNA, and its identification as a member of the TL family of PR proteins.

MATERIALS AND METHODS

Plant Material

The sweet cherry (*Prunus avium* L.) cv Bigarreau Summit was used throughout. Fruits were harvested at the green, straw-yellow (breaker), and ripe stages (36, 46, and 59 d postanthesis, respectively, at Avignon, France). Protein analysis and purification were done on fruit from the Avignon area; RNA analysis and cDNA construction were done on equivalent stages of fruit from Summerland, British Columbia, Canada. Several experiments included the additional stages of pink (between the yellow and ripe stages) and overripe (fruit harvested 2 weeks after the ripe stage). Freshly harvested fruits were frozen in liquid nitrogen after removal of stems and pits. For subsequent protein work, the tissue was stored at −20°C, and for RNA work, the tissue was freeze-dried and stored at −40°C.

Protein Extraction and Purification from Cherry Fruit

All steps were carried out at 4°C. To each 1 g of cherry tissue were added 2 mL of extraction buffer (0.2 M sodium acetate, 1.4 M sodium chloride, pH 5.5) and 50 mg of insoluble PVP previously hydrated in 0.5 mL of extraction buffer. After high-speed homogenization in an ice-cold Waring blender for 1 min and mixing with a magnetic stir bar for 1 h at 2°C, the slurry was passed through cheesecloth and centrifuged at 30,000*g* for 30 min. Pectic compounds were removed from the supernatant by precipitating at 4°C overnight in the presence of 0.04 M calcium chloride and then pelleting by centrifugation at 30,000*g* for 1 h. The purification continued with an 80% ammonium sulfate precipitation from this crude extract. The precipitate was redissolved in 0.1 M sodium acetate, pH 5, and dialyzed extensively against the same buffer. The proteins from 60 g of cherry tissue were chromatographed by size

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Abbreviations: PR, pathogenesis-related; TL, thaumatin-like.

exclusion (ACA 54 [IBF, Villeneuve la Garenne, France]; 100 × 1.6 cm, 185 mL bed volume; equilibrated and eluted at 9.6 mL/h with 0.1 M sodium acetate, pH 5) and anion-exchange (Pharmacia DEAE-Sepharose CL6B; 10 × 2 cm, 50 mL bed volume; the column buffer of 0.03 M sodium acetate, pH 5.0, was used for equilibration, sample loading, and initial elution at 60 mL/h). The distribution of the protein through the various chromatographic steps was monitored by SDS-PAGE.

Electrophoretic Experiments

Analytical SDS-PAGE was performed as described by Laemmli (1970) in a minigel apparatus (Bio-Rad). IEF analysis was performed with a Phast system (Pharmacia) using Phast gels IEF 3–9. Preparative SDS-PAGE was performed on a freeze-dried, purified protein using a minigel apparatus (Bio-Rad) with 1.5-mm-thick gel. The staining was done with Coomassie blue in water according to Harlow and Lane (1988). The protein band was excised and freeze-dried. Rabbits were immunized by Eurogentec (Seraing, Belgium).

Protein Characterization (Amino Acid Composition and Sequencing)

Protein sample was hydrolyzed in 6 M HCl in vacuo at 110°C for 24 h in the presence of 5% (v/v) phenol to prevent excessive degradation of Tyr. Amino acid analysis was performed on a Beckman 6300 amino acid analyzer.

Sequencing of protein was carried out on a gas-phase sequencer (Applied Biosystems 470 A) using the 03 RPTH program. Phenylthiohydantoin derivatives of amino acids were identified on-line on a phenylthiohydantoin analyzer (Applied Biosystems 120 A). The amount of protein submitted to Edman degradation was 1250 pmol.

RNA Extraction from Cherry Fruit

Total RNA was extracted from lyophilized fruit tissue by modification of the methods of Franklin (1966) and Morris and Dodds (1979). In brief, 15 g of dried tissue was powdered in liquid nitrogen with a Waring blender and then shaken for 45 min at room temperature in a mixture of: 45 mL of phenol:chloroform (1:1 [v/v], pH 7.6, equilibrated with Tris-HCl); 45 mL of extraction buffer (0.2 M Gly, 0.1 M disodium phosphate, 0.6 M sodium chloride, pH 9.5); 2.5 mL of 2-mercaptoethanol; and 2.5 mL of 20% (w/v) SDS. After centrifugation, the organic phase was reextracted with 15 mL of fresh extraction buffer. The aqueous phases were combined and reextracted with 50 mL of phenol-chloroform by vigorous shaking and by centrifugation. The nucleic acids in the aqueous phase were precipitated overnight with ethanol at −20°C and recovered by centrifugation. The resulting pellet was dissolved on ice in 100 mL of the buffer STE (0.1 M sodium chloride, 0.05 M Tris, 1 mM EDTA, pH 8.0), and ethanol was added to make a final concentration of 30% (v/v). Seven grams of cellulose CC 41 (Whatman) were added, and the mixture was shaken on ice for 45 min. The cellulose-bound RNA was washed extensively with STE containing 30% (v/v) ethanol, and the RNA was eluted with 0.5 mM EDTA. Northern hybridization blots were done according to the GeneScreen (New England Nuclear) instructions for formaldehyde gels.

Construction of the cDNA Library of Ripe Fruit

A λ Zap II (Stratagene) cDNA library was prepared from ripe cherry polyadenylic acid-containing RNA. The first strand was synthesized with Superscript reverse transcriptase (GIBCO-BRL) in the supplied buffer using 7.5 μg of template RNA and priming with 1 μg of oligo(dT) and 150 ng of random hexamers. The second strand was synthesized by *Escherichia coli* DNA polymerase in the presence of *E. coli* DNA ligase. *Eco*RI cohesive-end adaptors were ligated to the cDNA and then phosphorylated. Approximately 150 ng of the double-stranded cDNA was then cloned into the λ Zap II vector. The number of independent recombinants generated was 1.3×10^6. The average size of the cloned cDNA was estimated at 1 kb by PCR analysis of individual plaques.

λ Zap II cDNA Library Immunoscreening and Clone Analysis

Immunoscreening of the library was done according to the instructions of the manufacturer (*pico*Blue kit, Stratagene). Antibodies raised against the cherry 29-kD protein were preabsorbed to a nonrecombinant *E. coli*/phage lysate. Twenty positive clones were plaque-purified and subcloned by the Zap procedure (Stratagene). Sequencing used Sequenase (Amersham) according to the manufacturer's instructions. Both DNA strands of some cDNA clones of interest were fully sequenced using exonuclease III/S1 nuclease-generated nested deletions as well as specific internal primers.

RESULTS

Purification and Some Properties of the "29-kD Polypeptide"

Significant changes occurred in the protein profile of cherry fruit during ripening (Fig. 1A). Most notably, a polypeptide with an apparent molecular mass of 29 kD on SDS-PAGE increased dramatically in amount between the yellow and ripe stages. A small amount of this protein appeared to be present in the green fruit. The "29-kD polypeptide" eluted from a gel-filtration column at approximately 24 kD relative to protein standards and represented 38% of the total loaded material. When this peak fraction was applied to a DEAE column, a symmetrical peak that represented 52% of the loaded protein was eluted between approximately 2 and 3 column volumes. The 29-kD polypeptide was located in this peak. This elution behavior is consistent with the conditions of low ionic strength and pH 5, where a protein of pI 4.2 (Fig. 1B) would have a small charge retarding its elution.

Purity of the protein is demonstrated in Figure 1, B and C. Antibodies raised against the 29-kD band from preparative SDS-PAGE interacted strongly with a protein at 29-kD on western blots of both purified and crude extracts

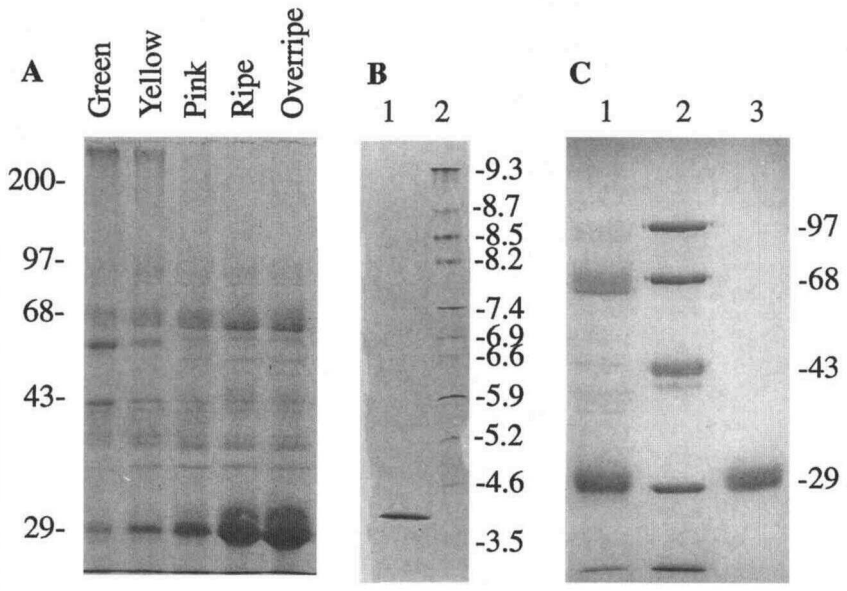

Figure 1. Electrophoresis of cherry protein extracts. A, Accumulation of the 29-kD polypeptide during ripening of cherry fruit. Extracts of cherry mesocarp at the indicated stages of fruit ripening were TCA precipitated, and 10 μg of protein from each were fractionated on a 10% SDS-PAGE. The molecular masses of protein standards in kD are given on the left of the figure. B, IEF of purified 29-kD polypeptide. The samples were applied in 1-μL volume to each lane. Protein pI standards were as marked. C, SDS-PAGE analysis of purified 29-kD polypeptide (lane 3) versus a crude extract from ripe cherry fruit (lane 1). Molecular mass standards are in lane 2. All gels were stained with Coomassie brilliant blue.

of protein (data not shown). They did not react with any other fractions from size-exclusion or ion-exchange chromatography (data not shown).

The amino acid composition of the purified protein revealed high levels of Asp + Asn (11.8%), Thr (9.6%), Gly (9.6%), Pro (9.4%), and Ala (9.2%). The 40 N-terminal residues of the polypeptide were determined to be: N-Ala-Thr-Ile-Ser-Phe-Lys-Asn-Asn-Cys-Pro-Tyr-Met-Val-Trp-Pro-Gly-Thr-Leu-Thr-Ser-Asp-Gln-Lys-Pro-Gln-Leu-Ser-Thr-Thr-Gly-Phe-Glu-Leu-Ala-Ser-Gln-Ala-Ser-Phe-Gln. The molecular mass of the purified 29-kD polypeptide was determined as 23,359 D by ion-spray MS.

Isolation and Characterization of the cDNA Clones

A cDNA library of ripe cherry was screened with the antibodies elicited with the purified 29-kD polypeptide. Twenty independent clones (pCHER29/1 to pCHER29/20) were thus isolated, and their estimated length ranged from 0.9 to 2.0 kb. Several of the largest clones were subsequently shown to have multiple inserts. All clones had a similar partial restriction map with the presence of internal *Kpn*I and *Bgl*II sites. Twelve clones of different lengths were partially sequenced; all contained exactly the same coding and 3′ noncoding sequences with the variation in length caused by transcriptional extension to include from one to up to four polyadenylation signals and variable length polyadenylic acid tracts.

The nucleotide sequence of clone pCHER29/6 has been deposited in GenBank database, accession no. U32440. An open reading frame of 245 amino acid residues was found between nucleotides 13 and 746. Comparing this deduced sequence to the sequence of the purified protein, an exact match was found starting with the Ala at position 24 of the deduced sequence. A short signal sequence of 23 amino acids preceded the mature protein composed of 222 residues. The predicted molecular mass of the preprotein was thus 25.8 kD, and the molecular mass of the mature protein was 23.3 kD, a difference of 2.5 kD. The pI calculated from the predicted mature protein was 4.18.

A search of the nonredundant peptide sequence database on the NCBI BLAST e-mail server (National Library of Medicine, Bethesda, MD) using the BLAST program (Altschul, et al., 1990) revealed a large family of related sequences. The protein family was referred to as "TL" or "PR class V" (Stintzi et al., 1993). All TL proteins have a high sequence homology with thaumatin, the intensely sweet-tasting protein of the *Thaumatococcus danielii* Benth fruit (Van der Wel and Loeve, 1972). The deduced amino acid sequence of the cherry 29-kD polypeptide was compared with the sequences of thaumatin and four representative TL proteins (Fig. 2). Protein sequence alignments were done using the program of Cabot and Beckenbach (1989). The mature cherry protein was 58, 41, 38, 41, and 38% identical, respectively, to: an *Arabidopsis thaliana* PR protein, tobacco osmotin, thaumatin, maize zeamatin (or α-amylase/trypsin inhibitor, Richardson et al., 1987), and tobacco mosaic virus-induced tobacco protein (Fig. 2, PR-S). Identification of the cherry TL protein was additionally supported by the cross-reactivity of our polyclonal antibodies with a commercial preparation of thaumatin (data not shown). The growth of the fungal pathogens *Botrytis cinerea* and *Monilia laxa* were not inhibited by 30 μg of the purified cherry TL protein in the assay conditions described by Huynh et al. (1992).

Northern Analysis

The expression of this protein appeared to be regulated by gene expression, with mRNA levels increasing through the pink into the full ripe stage (Fig. 3). Message levels appeared to decrease after full maturity, while the protein continued to accumulate. More than 2% of the inserts in our ripe cherry cDNA library hybridized to a DNA probe from the 5′ end of the cherry TL clone. This was consistent with a high level of expression in the ripe fruit.

Figure 2. Alignment of TL proteins. The cherry TL protein (CHTL) was compared to: ARTL, an *A. thaliana* TL protein (Uknes et al., 1992); OSMO, osmotin, a protein of NaCl-adapted tobacco cells (Singh et al., 1989); THAU, thaumatin from *T. daniellii* (Edens et al., 1982); ZEAM, zeamatin: antifungal protein from maize seeds (D.M. Malehorn, J.R. Borgmeyer, C.E. Smith, J.G. Layton, D.M. Shah, unpublished data, GenBank accession no. U06831); and PR-S, a virus-induced protein from tobacco (Cornelissen et al., 1986). Amino acid residues identical to the cherry TL are darkly shaded. Blank spaces have been introduced to allow improved alignment of homologous regions. The numbers in the right margin indicate the sequence position for each protein. The amino acid residues in bold in the cherry sequence identify the sequence found in the mature, purified protein with the 1 showing start of the mature protein. The 16 conserved Cys residues are boxed. The numbers 1, 2, and 3 under the last sequence indicate the different domains of the thaumatin protein.

DISCUSSION

A protein present in only small quantities in green fruit rapidly accumulates until it is the most abundant of the total soluble proteins in ripe cherry fruit. Antibodies elicited by this purified protein were used to identify corresponding cDNA clones. Sequence information derived from these cDNA clones suggested that this significant protein in ripe cherry is a member of the thaumatin family.

The characteristics of the deduced protein encoded by pCHER29/6 corresponded very well to the physical properties of the purified protein. The molecular mass by mass spectroscopy, the amino acid composition, the pI, and the N-terminal amino acid sequence of the purified protein were the same as predicted by the cDNA sequence. One characteristic that is not understood is the migration of the purified protein at 29 kD on SDS-PAGE instead of the predicted 23.3 kD. However, the antibody preparation reacted on western blots with the major protein at 29 kD, and no signal was apparent at lower molecular mass, which suggests that the cDNA clones were not incorrectly identified by contaminating antibodies. In addition, the anomalous migration of PR proteins in SDS-PAGE has been previously reported (Cusack and Pierpoint, 1988). The abundance of this protein, its apparent molecular mass, and its amino acid composition suggest that this is the same protein that Krishnan and Pueppke (1990b) reported as having antigenic homology with deglycosylated β-fructosidase from carrot. However, the pCHER29/6 sequence had no homology to published glycosidases, although antigenic similarity cannot be excluded.

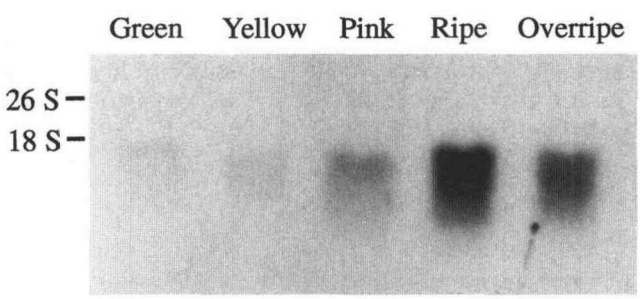

Figure 3. Cherry TL gene expression during ripening of cherry fruit. Ten micrograms of total RNA from five stages of cherry fruit development were analyzed by agarose gel electrophoresis and blotted onto a nylon membrane. The blot was hybridized to a ^{32}P-labeled probe of the insert from pCHER29–6 and washed at high stringency. The autoradiogram was exposed for 4 h.

The TL proteins are divided into two subgroups: a basic form found in the vacuole and an acidic form that is apoplastic (Stintzi et al., 1993). The presence of a signal peptide, the absence of a C-terminal extension as found in vacuolar forms, and the acidic character of cherry TL suggested that this protein is apoplastic (Bol et al., 1990). This was consistent with the results of Krishnan and Pueppke (1990b), which showed that the 29-kD polypeptide was localized in the cell wall.

A common function associated with a number of the TL proteins has been an antifungal activity, possibly mediated by membrane permeabilization (Roberts and Selitrennikoff, 1990; Vigers et al., 1991, 1992). The sequence of the cherry TL protein has the characteristics thought to be important for this function. Particularly, the 16 Cys residues involved in the disulfide bridges of thaumatin were conserved among the 6 TL proteins (Van der Wel et al., 1984). These appeared to be important in maintaining the β-sheet structure in domain 1 as determined in the crystal structure of thaumatin (Van der Wel and Ledeboer, 1989). The cherry peptide is longer than most of the TL proteins. The additional amino acids were located predominately in domains 2 and 3, which are in loops exterior to the β sheet. The other residue additions occur where turns in this core structure were predicted and again would not be likely to

interfere with this structure's formation. The cherry TL protein accumulated in fruit, so it could be expected to have the sweet taste associated with thaumatin. However, it was similar to other TL proteins in this regard, with no apparent sweetness to humans.

The expression of the cherry TL protein appeared to be regulated by gene expression with mRNA levels increasing through the pink into the fully ripe stages. This is consistent with the response during ripening of a ripening-related gene but also with the response of a PR gene to infection. The high level of expression of this protein has been observed in all varieties of cherries tested in both Europe and North America (data not shown), so it seemed unlikely that a pathogen could be responsible for this uniform response. There is evidence for developmental regulation of TL proteins in other tissues, including tobacco floral tissues (Richard et al., 1992); antifungal proteins in the seeds of wheat, barley, and maize (Roberts and Selitrennikoff, 1990); and the arils of the fruit of *Thaumatococcus* (Mackenzie et al., 1985). TL PR proteins, like other PR proteins, are induced by a number of common plant chemical signals, including ethylene and ABA, both also known to have a role in ripening responses. It is uncertain what stimuli are involved in ripening in nonclimacteric fruit, such as cherry, but the present data suggest a similarity between the ripening process and the induction of the PR response.

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