cDNA sequence and heterologous expression of monomeric spinach pullulanase: multiple isomeric forms arise from the same polypeptide

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The spinach pullulanase gene was cloned and sequenced using peptide sequences of the purified enzyme as a starting point and employing PCR techniques and cDNA library screening. Its open reading frame codes for a protein of 964 amino acids which represents a precursor of the pullulanase. The N-terminal transit peptide consists of 65 amino acids, and the mature protein, comprising 899 amino acids, has a calculated molecular mass of 99 kDa. Pullulanase is a member of the α -amylase family. In addition to a characteristic catalytic (β/α)₈-barrel domain, it contains a domain, F, that is specific for branching and de-branching enzymes. Pullulanase cDNA was expressed in *Escherichia coli*, and the purified protein was compared with the enzyme from spinach leaves. Identity of the two proteins was confirmed in terms of catalytic properties, N-terminal amino acid sequences and molecular masses. The pullulanase produced by *E. coli* showed the same microheterogeneity as the spinach

leaf enzyme: it could be resolved into two substrate-induced forms by electrophoresis in amylopectin-containing polyacrylamide gels, and, in the absence of substrate, into several free forms (charge isomers) by isoelectric focusing or chromatofocusing. Rechromatofocusing of single free forms resulted in the originally observed pattern of molecular forms. However, heterogeneity of the protein disappeared on isoelectric focusing under completely denaturing conditions when only one protein band was observed. Post-translational modifications such as glycosylation and phosphorylation could be excluded as potential explanations for the protein heterogeneity. Therefore the microheterogeneity of spinach leaf pullulanase results from neither genetic variation nor post-translational modifications, but is a property of the single unmodified gene product. The different interconvertible forms of the pullulanase represent protein populations of different tertiary structure of the same polypeptide.

INTRODUCTION

The pullulanase (R-enzyme) from spinach leaves is a chloroplastic monomeric starch-debranching enzyme which shows a special kind of protein polymorphism. It separates into two 'substrate-induced' forms on amylopectin-containing PAGE [1] and into up to seven isomers on isoelectric focusing [2]. The isomers are antigenically related and can be mutually interconverted [3]. This microheterogeneity was interpreted as coexistence of different conformational isomers of a single polypeptide which is apparently stable in different tertiary structures [3]. This differs from the polymorphism of other starch-degrading enzymes. The different forms of starch phosphorylase from spinach or potato, for example, are immunologically distinguishable, located in different cell compartments and have been related to different genes [4]. Several forms of β -amylase from wheat leaves are immunologically related and are derived from a single protein which post-translationally undergoes partial proteolysis [5]. Other examples of protein heterogeneity caused by post-translational modifications are protein aggregation of α -amylase from *Vigna mungo* seeds [6], protein glycosylation of polyphenol oxidase from *Vicia faba* [7] and different phosphorylation levels of many enzymes, e.g. nitrate reductase and sucrose phosphate synthase [8].

Confirmation of the model proposed by Henker et al. [3] for the coexistence of different conformational isoforms of the pullulanase requires exclusion of all the modes described above that lead to protein heterogeneity. In the present paper we show

that all isoforms of the pullulanase are translation products from a single mRNA. For that purpose we cloned the cDNA from spinach leaves [9] and expressed it in *Escherichia coli*. The recombinant protein was compared with the enzyme purified from spinach leaves with respect to kinetic and biophysical properties as well as microheterogeneity and interconversion of individual isoforms. Furthermore we show that post-translational protein modifications can be excluded as mechanisms leading to the protein polymorphism. Finally, biochemical consequences of the microheterogeneity of the pullulanase are discussed.

EXPERIMENTAL

Materials

Spinach plants, chemicals, biochemicals and other items were obtained as described previously [3]. All radioisotopes were from DuPont-NEN, except [α -³²P]dCTP which was from ICN. The DNA-labelling kit, restriction enzymes and general molecular-biology reagents were from Boehringer (Mannheim, Germany). The Sequenase version 2.0 DNA sequencing kit was from Upstate Biotechnology/Life Technologies, and the 'uni-ZAP XR cDNA library kit' was from Stratagene. Hybond-N nylon membranes for library screening were obtained from Amersham; the sure clone ligation kit was from Pharmacia.

Spinach leaf pullulanase was purified as described by Ludwig et al. [1]. Chromatofocusing, SDS/PAGE, isoelectric focusing

Abbreviation used: MALDI-TOF-MS, matrix-assisted laser-desorption/ionization time of flight mass spectroscopy.

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and assays for pullulanase activity and protein were as in Henker et al. [3].

Sequencing of CNBr fragments

Internal fragments of pullulanase were prepared by digesting 80 µg of purified lyophilized protein in 80 µl of 70% formic acid with 17 mM CNBr overnight in the dark at room temperature [10]. The sample was diluted 10-fold in water, lyophilized, washed twice with water, lyophilized again, and dissolved in SDS-loading dye. Peptides were electrophoretically separated in 12–18% SDS/polyacrylamide gels containing 6 M urea [11,12], transferred to a PVDF nylon membrane by semidry blotting [13], and then stained with Amido Black. The stained peptide bands were excised and subjected to automated Edman degradation in a pulsed-liquid sequencer (Applied Biosystems; model 473A).

Cloning and sequencing of pullulanase cDNA

From the peptide sequences obtained, various oligonucleotides were designed for use in PCR using a codon usage table for spinach prepared by the program CODONFREQUENCY of the GCG software. Two of the primers had the sequences 5'-AA(T/C)AT(T/C)GATGG(T/A)GTIGA(A/G)GG-3' (sense encoding NIDGVEG) and 5'-AT(A/T)GT(T/C)TC(A/G)-AT(A/T)GG(T/C)TGCAT-3' (antisense encoding MQPIDTI); the parentheses indicate that at the third position of these triplets a mixture of the listed bases was used for primer synthesis; I means inosine. A 100 pmol amount of each primer was used. PCR conditions were 1 min at 95 °C, 1 min at 52 °C, and 1 min at 72 °C for 30 cycles followed by 72 °C for 10 min. cDNA derived from total RNA of 6-week-old spinach leaves was used as a template; Mg²⁺ concentration was 1.5 mM. PCR products were gel-purified before cloning into pUC18.

The subcloned cDNA was labelled with [α -³²P]dCTP by random-priming and used as a probe to screen a spinach leaf cDNA library in λ ZAPII. Nylon membranes were probed with labelled cDNA at 42 °C overnight in buffer containing 25% (v/v) formamide, 250 mM sodium phosphate, pH 7.2, 250 mM NaCl, 1 mM Na₂EDTA, 7% (w/v) SDS, 25% (w/v) poly(ethyleneglycol) 6000 and 0.25 mg/ml sheared salmon sperm DNA. After hybridization, the membranes were washed once in 1 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% (w/v) SDS, and twice in 0.1 × SSC containing 0.1% (w/v) SDS at 42 °C. Positive clones were purified by three further rounds of screening. Insert cDNAs were excised into pBluescript II SK (Stratagene) according to the manufacturer's instructions. The largest cDNA clone was chosen for DNA sequencing and was shown to be the 3' end of the pullulanase gene. No full-size clones for the pullulanase gene were found in the λ ZAPII library.

A second cDNA library was prepared from 1.0 mg of total RNA which had been extracted with tip-500 columns from Qiagen (Hilden, Germany) from 1.0 g of 6-week-old spinach leaves. Polyadenylated mRNA was isolated using Dynabeads MPC-1 with covalently coupled oligo(dT)₂₅-DNA (Dyna, Hamburg, Germany) [14]. Polyadenylated mRNA (5 mg) was transcribed into cDNA using *Xho*I-oligo(dT)₁₈ primers. After addition of *Eco*RI linkers, the cDNA was digested with *Xho*I and was size selected by gel filtration with Sephacryl S-400 columns (Pharmacia). cDNAs larger than approx. 0.7 kb were ligated into *Eco*RI-*Xho*I-digested dephosphorylated λ uni-ZAP XR vector-arms (Stratagene) and packaged using the 'gigapack II gold packaging extract' (Stratagene). The cDNA library was screened according to the manufacturer's instructions. Hybridization and washing of membranes and the *in vivo* excision of inserted

cDNAs into pBluescript II SK were as described above. The largest cDNA clones were chosen for DNA sequencing and were shown to be full-size clones of the pullulanase gene. Manual sequencing of both DNA strands was carried out using the Sanger dideoxy termination method provided by the Sequenase version 2.0 kit. Sequence alignments were made using the programs FASTA, BESTFIT and PILEUP (from GCG).

Expression of spinach pullulanase cDNA in *E. coli*

The part of the pullulanase gene coding for the mature protein including the 3' untranslated region was excised from pBluescript II SK by *Nco*I-*Xho*I digestion and was cloned into the *Nco*I-*Xho*I sites of the expression vector pET-15b (Novagene). The construct termed pET-RE7 was analysed by restriction enzyme digestion and DNA sequencing to confirm sequence fidelity.

For large-scale purification of recombinant pullulanase, plasmid DNA was transferred to the *E. coli* strain BL21(DE3) (Novagene). Cells were grown overnight at 37 °C in the presence of ampicillin (100 mg/ml). The culture was then diluted 1:50 and grown at 27 °C. When an A_{600} of 0.7 was reached, expression of the pullulanase gene was induced with isopropyl β -D-thiogalactoside (final concentration 1.0 mM) and the cells were maintained at 27 °C for another 4 h. Cells were spun down at 4 °C, and frozen at -20 °C. The pellet was resuspended in lysis buffer (50 mM Tris/HCl, pH 8.3, 1 mM Na₂EDTA, 50 mM NaCl, 1 mM PMSF), and the suspension was treated with lysozyme and DNase I [15]. Cells were finally lysed by three passages through a french press. The pH was adjusted to 5.8 and the homogenate was centrifuged at 50000 g for 30 min. The supernatant was applied to a β -cyclodextrin-Sepharose 6B column [1] equilibrated in sodium acetate buffer, pH 5.8. After washing with 2 litres of the same buffer, pullulanase was eluted with sodium acetate, pH 5.8, containing 5 mg/ml β -cyclodextrin. Cyclodextrin was removed by ultrafiltration in centricon 50 tubes (Amicon), and the purified protein was stored at -20 °C.

In vivo labelling of phosphoproteins in *E. coli*

To analyse potential phosphorylation of recombinant pullulanase, a culture of *E. coli* BL21(DE3) transformed with the pET-RE7 plasmid was grown in phosphate-depleted LB medium [16] containing ampicillin. Concomitantly with isopropyl β -D-thiogalactoside, 2 mCi of [³²P]P_i was added to the culture. After 4 h, cells were sedimented and extracted as described above.

Glycoprotein detection

The glycoprotein character of spinach pullulanase was analysed using the 'DIG glycan detection kit' (Boehringer) according to the manufacturer's instructions.

CD spectroscopy

CD spectra were recorded using a Jasco J-600 spectropolarimeter. Far-UV-CD spectra (180–250 nm) were recorded in 10 mM sodium phosphate buffer, pH 7.0, at a resolution of 0.2 nm, and ten spectra were accumulated. The speed and time constants were 20 nm/min and 1 s respectively. The sensitivity was 10 milli-degrees and the bandwidth 1 nm. The pullulanase concentration was 20 µM. The molar CD absorption coefficient $\Delta\epsilon$ was calculated from the measured ellipticity [θ] using eqn. (1) [17]:

$$\Delta\epsilon = \frac{[\theta]100 \text{ MW}}{cd3300} \quad (1)$$

where MW is the molecular mass, c is the concentration and d is the optical pathlength.

Fluorescence spectroscopy

Fluorescence measurements were performed at 25 °C in an Hitachi F-4500 spectrofluorimeter. Excitation and emission wavelengths were 280 and 320 nm respectively. To measure a urea-induced transition, the pullulanase was incubated for 16 h at 25 °C in piperazine/HCl, pH 6.0, containing 0–10 M urea.

MS

Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed in a MALDI II mass spectrometer (Shimadzu, Japan). Sinapinic acid was used as a matrix. A 0.2 mg/ml sample of pullulanase was dissolved in 10 mM Tris/HCl, pH 7.0. BSA was used as standard.

Isoelectric focusing under denaturing conditions

The 7.5% acrylamide gels contained 10 M urea, 2.5% Servalyt 5–7 and 2.5% Servalyt 7–11 (both from Serva). Before focusing, pullulanase samples were incubated for 16 h at 25 °C in 10 mM piperazine/HCl, pH 6.0, containing 10 M urea.

RESULTS

Peptide sequence data of the spinach pullulanase

Cleavage of the purified spinach leaf pullulanase with CNBr followed by SDS/PAGE and protein blotting yielded seven distinct peptides ranging in size from 9 to 55 kDa (results not shown). Amino acid sequence analysis of the individual peptides gave unambiguous sequences of three peptides (Table 1). The other bands either contained more than one peptide or appeared to be partial digestion products.

Isolation of cDNAs encoding spinach pullulanase

The peptide sequences obtained were used to design redundant oligonucleotide primers. With spinach leaf cDNA as a template, two of the primers (sequences given in the Experimental section) yielded a single product (420 bp) on PCR. The subcloned PCR product contained sequences corresponding to peptides 2 and 3. The 420 bp cDNA was used to screen an oligo(dT)-primed spinach cDNA library in λ ZAPII. One positive clone, designated RE1, was obtained from this screen with an insert size of approx. 1.6 kb. It contained a single open reading frame including the sequence of the PCR product and the 3' end of the pullulanase gene; however, the 5' end of the gene was missing.

RE1 was digested with *Pst*I and the 5' end (1.2 kb in size) was used as a probe to screen a λ uni-ZAP XR cDNA library from spinach leaves. Of 1.3×10^6 clones, there were four positive clones with an insert size of more than 3.3 kb. They all contained the complete coding region of the pullulanase gene. Two of them, however, included intron sequences. The other two clones, designated RE4 and RE7, with an insert size of 3.4 kb, were identical and were shown to derive from a correctly spliced mRNA containing the complete coding sequence plus the 5' and 3' untranslated region of the pullulanase gene [9].

Nucleotide sequence of RE7 and the deduced amino acid sequence

The nucleotide sequence of RE7 consists of an open reading frame of 2892 bp. It has a 200-nucleotide 5' untranslated region

Table 1 Partial amino acid sequences of CNBr fragments of spinach pullulanase

Sequences were obtained from spinach leaf pullulanase as described in the Experimental section. The parentheses indicate that methionine is assumed to be immediately N-terminal to the sequenced peptides since they were derived by CNBr cleavage; X means unidentified amino acid.

Peptide	Sequence
1	(M)AVEVGSASXVXQXEL
2	(M)LQGLXKNIDGVEGXSIL
3	(M)NPIETINYVSAHXDNE

and a 345-nucleotide 3' untranslated region including a poly(A) tail of 44 A residues.

A putative polyadenylation signal motif (AATAAA) was found 90 bp downstream of the stop codon TAA (Figure 1). The open reading frame encodes a 964-amino acid polypeptide with a molecular mass of 106 kDa [9]. Edman degradation of pullulanase purified from spinach chloroplasts showed that the N-terminus of the mature protein is Ala-66. The 65 amino acid transit peptide shares some features with other chloroplast transit peptides, e.g. a high content of Ser, few acidic residues [19] and Ala in position +1 of the transit peptide cleavage site [20]. The mature protein has a predicted molecular mass of 99 kDa, which corresponds closely to the data for purified spinach pullulanase [1]. The predicted amino acid sequence contains the sequences derived from Edman degradation of CNBr fragments of the protein. Sequence comparison with other starch-degrading enzymes showed high similarity to the pullulanases of *Klebsiella aerogenes* and *Bacillus stearothermophilus* (58%). Meanwhile a sequence of rice pullulanase has been published [21] that shows 80% identity with the spinach enzyme.

Structural predictions for the pullulanase

Together with α -amylases, α -glucosidases, cyclodextrin-glucanotransferases and isoamylases, pullulanases belong to family 13 of the glycosyl hydrolases [22,23]. Sequence comparison shows that the mature spinach pullulanase shares a catalytic domain A with all members of this protein family. Upstream of the catalytic domain is domain F, which is typical of branching and debranching enzymes [24]. The N- and C-terminal parts of the pullulanase show only a few similarities to other members of this protein family (Figure 2).

On the basis of three-dimensional studies of some α -amylases and cyclodextrin-glucanotransferases, the secondary structure of catalytic domain A was predicted for some members of the α -amylase family as a $(\beta/\alpha)_8$ -barrel [25]. Secondary-structure prediction using the Chou and Fasman algorithm [26] showed that the catalytic domain of spinach leaf pullulanase forms a $(\beta/\alpha)_8$ -barrel which contains an extraordinarily long loop between β -strand 2 and α -helix 2 (results not shown).

Expression of the cDNA encoding mature pullulanase RE7 in *E. coli*

The pullulanase cDNA was expressed in *E. coli* BL21(DE3) after transformation with pET-RE7. Crude extracts of induced cells contained pullulanase with a specific activity of 0.14 unit/mg of protein (Table 2), whereas no activity was detectable in control cells containing the plasmid pET-15b (results not shown). The recombinant pullulanase (RE7) was purified to homogeneity by affinity chromatography and analysed by SDS/PAGE. It migrated as a single protein band with the same electrophoretic

1 AAAACATTCGATAGGCGCAAAATAACAAACCCCAAACTCTAACCATGAATCTCATCTTTTAAACATCA
75 TTTTTCATCGAAATCTAGCTTCTGTAACTAATTTTCCCACTTTACAGCATCATCTTCATCTGCTCAACTGAAT
150 TTCTGCTTAAACCGCCATAGCCAAAACTCAACCTCACATTTATGCTCTAATGCTCTCACTATATAACCCCAT
M S S L Y N P I 8
225 GCTCTGCTCTAGTTTCCATCACCATTCTCTAATCTTGGTTTCTACCTTTAATTTCAATTTTATACCAAA
A L A S S F H H H Y P N L R F L P F N F N F I T K 33
300 TTACCCGTTCTTAATCTCTTGTATTTGGGTTCTAGTTCTAGAGGCTTCCATTCATCGCCATGAAGAAGATTCT
L P V S N S F A I T P N V T E K F P H I R G V S A F 58
375 TCTTGTCTTTGTGTTCCATGGCTGCGAAGTTGGTTCTGCTCTCTGTTTCTCAGAGTGAATTCGAAGGAAT
S C P C C S M A V E V G S A S S V S Q S E L Q G S 83
450 TTGAATAGTTGTAGAGCTTATGGCTAGCAGTATACATTTGCTGGAATGTTGATATTGGTATGGTTCATAT
L N S C R A Y W P S K Y T F A W N V D I G N G S Y 108
525 TACTTATTTGCAAGTAAAGCTGCTCCCTTAAAGTTTACAGATCTGGGATAGAAGATACGACGTGAATCAAG
Y L F A S K T A A L K F T D A G I E G Y D V K I K 133
600 CTTGACAAGCAACAGGGGATTTGCCAGCAATGCTCACTGAAAAATTTCTCATATAGAGTTACTGGGCTTT
L D K D Q G G L P A N V T E K F P H I R G V S A F 158
675 AAAGCTCCAGCAGCATGAGTGTGATAGTCTGCTGAGAGTGAACCTGCTGCTTCTCAGTCTGACGGG
K A P A T L D V D S L L K C Q L A V A A F S A D G 183
750 GCTTGCAGAAATGCTACTGGTTTGCAGTTGCTGGGTTTATGATAGTGTGATTCATATGAGCCCTCTGGGT
A C R N A T G L Q L P G V I D E L Y S Y D G P L G 208
825 GCTCTTTCTCAGAAACACCATATCACTGTACCTATGGGCTCTCTGCTCAAGCTGTTTCTGCGACATATTT
A V F S E N T I S L Y L W A P T A Q A V S A S I F 233
900 AAGGATCCATCAGGTGGTGAACCATTACAAACCTGCGAGCTTATAGAGTCAATGGTGTGGAGCCCTGTGGG
K D P S S G G E P L T V Q L I E S N G V W S A F 258
975 CCAAGAACCTGGGAGGGGTGTATTTATGTTTATGAACTCACTGTCTATCAACATAGCACCTTGAGAATTAAGAAA
P R T W E G C Y H V Y E I T V Y H S T L R I E K 283
1050 AGCTTTGCTATTGATCCATATGCCAGAGGATTTGAGCTGATGAAGCAACATTTTGGCTGACTTAAGCTCT
S F A I D P Y A R G I S A D V K R T L L A D L S S 308
1125 GAACTCTTAAAGCTGGAAGATGGGAAATCTTGGTGAATGAAAACTCACTCTTCTTCCATCTGACATCAGT
E T L K P E G W E N L A D E K P H L L S P S D I S 333
1200 CTCTATGAGCTCATATAAGAGATTTCAGTGTCTATGACCTCACTGTGACCCCTGACCTTGGTGGATCTT
L Y E L H I R D F S A T T V H P D L R G G A Y L 358
1275 GCTTTCACCTCAGAGGCTCAGCTGGTGTATATCTTGAAGTATCTGCTGCTGGTCTTACTCAGCTCAT
A P T S Q D S A G Y N H L E K L S A A G L T H V H 383
1350 CTGCTGCCAAGCTTCCAGTTTGTGCTAAGTGTATGATGACAAAAAGAGTGAATTTGTTGATCAAGAGGTT
L L P S F Q F A E V D D D K K K W K F V D T K R F 408
1425 GAAACATCAACCTGATTTCAGAAAGACACAGCTCAAAATCACTGATCCGAGATGAAGATGATTAACCTGG
E T L P P D S E E Q Q A Q I T A I R D E D G Y N W 433
1500 GGGTATAATCTGTTTGTGGGGAATCTTGAAGGAAGTATGCAACAGATCCAAATGGTCCATCCGATATAAT
G Y N P V L W G T P K G S Y A T D P N G P C R I I 458
1575 GAGTTCAGAAAGATGCTCAGGCGCTAAATCTGATTTGCTTCCGCTAGTTTGGATGTTTGTATTAACATTTA
E F R K M V Q A L N R I G L R V V L D V V Y N H L 483
1650 AATAGCAGTGGGCTCCGATGATAATTTCTGCTGGAAGATTTGCTCAGGTACTACTTAAGAAGAGATAAT
N S S G P S D D N S V L D K I V P G Y Y L R R D N 508
1725 GATGGTGTATTAATGAATGACATGCTGATGACACCTAGCAGCATTTTATGGTTGAAGCGCTGATTTTG
D G A I E N S T C V N D A S E H F M V E R L I L 533
1800 GATGATCTAAACATTTGGCGGCTGAATTAATAGGTTGATGGTTTCAGATTGATCTTATGGGCCATTAAGAA
D D L K H W A V N Y K V D G F R F D L M G H I M K 558
1875 CATACAGTGTGAAAGCGCAAAATATCTCTCAAGCGCTGTCAAAAACATAGATGGTGTAGAGGTTTCAAGCAT
H T M V K A T N M L Q G L S K N I D G V E G S S T 583
1950 TATTTATATGTAAGATGGGACTTTGGCAGGTGGCAATTAATGCACTGGAATTAATGCACTCACTGAAT
Y L Y G E G W D F G C E V A N N A R G V N A S Q L N 608
2025 CTGAGGAAACAGAAATGGAAGTTTAAATGATCGAATTCAGATGCACTGCTTGGTGGGCTCTTGGTCCC
L G G T G I G S F N D R I R D A V L G G G P F G P 633
2100 CCTCTCAGCAAGGTTACGTGCTGGTTTATCTTTACAGCTTAATGATCATGACCATAGCGGTAAGCAATGCA
P L Q G G Y V T G L G L S L Q P N D H D H S G K N A 658
2175 GACGATGCTGCTGCTGCGCAAAAGATCATACAGTTGGGATGGCTGCAAACTTGAGAGACTACATCTGACA
D R M L A V A K D H I Q V G M A G N L R D Y I L T 683
2250 AACTGTATGAGAAACAGGTGAAGGCTCAGAAAGTTATACCTATGGGGGAACCGCGTGGGTATGCTATGAC
N C D G K Q V K G S E V Y T Y G G T P V G Y A M Q 708
2325 CCGATAGAACTATCACTATGCTCAGCTGATGACAGCAAGCTTTTCGATATGCTATGAGAGCTCT
P E T I N Y V S A H D N E T L F D I V S L K T P 733
2400 ACCTACATACGTTGATGAGAGATGATGAGGTAAATCATTTAGCTACGAGTATTTAGCACTTTCCAGGGAATA
T Y I T V D E R C R V N H L A T S I L A L S Q Y I 758
2475 CCCTTTTCCATGCTGCTGATGAGTTGCTGACGTTCAAGGCTCCCTGACCGTGATCTTATAACTCTGGTATGG
P F F H A G D E L L R S K S L D R D S Y N S G D W 783
2550 TTTAACAGATTAGACTTCAGCTATACTCCAAATTTGGGTTGTTGCTCTCCCTCCCAAGGATCAATAGAGC
F N R L D F S Y N S N N W G V G L P P K D H N E S 808
2625 AATGGCCATTAATCAAGAAAGATGGCAATCCGCTCAAGGCTGACAGAAATCACATTTTGTGCTGCTT
N W P L I K K R L A N P S K Y P D K N H I A A V 833
2700 GAAAAATTCACCAATTTGTTGCAAAATGAGATCTCTTCCACTATTCGTTTAAAGAGTGCAAGAGATTTGAG
E N F T N L L Q I R Y S S P L F R L R S A K D I E 858
2775 GATGAGTACGATTCACCAATATGTTCCATCTTGGATTCTGGGCTTATAGCTATGAGCATTAAGATGGTCAT
D R V R F H N N V P S W I P G L I A M S I E D G H 883
2850 GCGGAGCCCTGGCTGTGACAGATAGATCCCAAGTTCAGTACATTTGTTAATCAATGATGCTCAGCTTACT
A G A P G L S Q I D P K F Q Y I V V I I N V Q P T 908
2925 GAAACCAATTTGTTAACCCAGATCTCGAGCTAAATCCCTACAGCTGCATCCAGTACAGTCAACATCAGGGGAC
E T K F V N P D L R A K S L T H P V Q S T S G D 933
3000 ACGTTGTAAAGAAATCAAGTATAGGCTTCTACTGAGTGTCTTACTATACCTCCTAAATCACTGCAATGCTC
T V V K E S K Y E P S T G C F T I P P K S T A V F 958
3075 GTTGAGCCACGGCATGTTTAAAGCTGAAGTTGAAGGCTGTGTCAGACGGCAGCGCATGTGGTTGTGCAAGT
V E P R H V * 964
3150 GGAATTACTTCTGCAATTTACACGGTTCAATACAAATAAAGCTCTGCTACCGCAGCGAGCTGAGGTCTCAC
3225 AGAATAGTTTACAAAAAAGTTACAGATTTATATTTTCAATAGCTCATATGTCAGCTGAATAAGAACCAAAAATCT
3300 TACGTTGACTTGTAGCAGTCTTTTGTGACGCATAAATACAGTTGCTTGTAAACATACATCCGATATGAAT
3375 GAAATAATTTTTTTTTTTTTTAAAAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

mobility as the enzyme from spinach (Figure 3). Using the pET-system, 9 mg of RE7 could be purified from 60 g of *E. coli* cells, which is a 380-fold higher yield than the preparation of pullulanase from spinach leaves (Table 2). The yield could be further increased by a factor of 9 when the 3' untranslated region of the pullulanase cDNA was removed and the RE7 cDNA was cloned into pET-15b (results not shown).

Comparison of the recombinant RE7 protein and spinach pullulanase

The proteins purified from *E. coli* and spinach exhibited similar specific activities (39 and 35 units/mg protein respectively) and the same substrate specificities. Also catalytic properties such as apparent K_m and V_{max} as well as competitive inhibition by β -cyclodextrin were very similar (Table 3).

The N-terminal sequence of the recombinant pullulanase was determined as AVEVGSASS, which was identical with the results for mature pullulanase from spinach chloroplasts. Obviously *E. coli* removed the fMet which should be the N-terminus of the translated protein.

To confirm the identity of the recombinant and spinach pullulanases, MALDI-TOF-MS was carried out. The two proteins showed very similar molecular masses of 99.9 and 99.8 kDa respectively (Figure 4). Features of the secondary structure were investigated by CD spectroscopy of the purified pullulanase from *E. coli* and spinach. The ratio of secondary-structure elements was calculated by the program variable selection [27,28], which compared the pullulanase CD spectra with those from proteins of known structure. The two pullulanases showed identical CD spectra (Figure 5) and a high proportion of β -sheet as the major secondary-structure element (Table 4).

Examination of microheterogeneity of RE7 protein

On electrophoresis in an amylopectin-containing polyacrylamide gel, the recombinant pullulanase and the protein from spinach separated into the two substrate-induced forms II and III (Figure 6).

Microheterogeneity of the recombinant pullulanase in the absence of substrate was examined by isoelectric focusing and chromatofocusing. The recombinant pullulanase as well as the original spinach enzyme produced several protein bands in the pI range between pH 4.7 and 5.2 (Figure 7). The individual molecular forms of the RE7 protein exhibited the same pIs as those of the spinach pullulanase. The proportions of the molecular forms, however, were not identical. The spinach protein showed two major forms whereas only one form dominated in the pattern of RE7 (Figures 7 and 8).

The interconvertibility of the isomeric forms of the recombinant pullulanase was examined by chromatofocusing on a Mono P column connected to an FPLC system. Up to nine forms in the pI range from 4.7 to 5.2 with different specific activities could be separated (Figure 8). Single fractions from this chromatofocusing experiment were collected, the buffer was changed

Figure 1 Molecular cloning of pullulanase cDNA RE7

The nucleotide sequence of the RE7 cDNA insert and its deduced amino acid sequence are shown. Both strands of the cDNA insert were sequenced using the dideoxy chain-termination method [18]. The upstream in-frame stop codon (TGA) in the 5' untranslated region and the consensus polyadenylation signal in the 3' untranslated region are underlined. The internal CNBr peptide sequences are marked. The chloroplastic transit peptide cleavage site is indicated by an arrow.

Figure 2 Structural predictions for the spinach pullulanase

The schematic diagram compares the domains of spinach pullulanase and other starch hydrolases. RESo, pullulanase from *Spinacia oleracea*; PuKa, pullulanase from *Klebsiella aerogenes*; PuBs, pullulanase from *Bacillus stearothermophilus*; IaPa, isoamylase from *Pseudomonas amyloidermosa*; BEEc, branching enzyme from *E. coli*; αAAo, α-amylase from *Aspergillus oryzae*. See the text for definition of domain A, C and F (modified from [24]).

Table 2 Purification of RE7 and pullulanase from spinach leaves

Spinach (500 g fresh weight) was grown in hydroculture, and *E. coli* BL21(DE3) (60 g fresh weight) was grown in a 9-litre fermenter; the purification procedures were carried out as described in the Experimental section. β-CD-eluate, eluate from affinity chromatography; 40–50% (NH₄)₂SO₄, resuspended pellet after (NH₄)₂SO₄ precipitation.

Source	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Yield (%)	Enrichment (fold)
<i>E. coli</i> BL21(DE3)					
Crude extract	2800	390	0.14	100	1.0
β-CD-eluate	9	350	39.00	90	280.0
Spinach leaves					
Crude extract	1900	40	0.022	100	1.0
40–50% (NH ₄) ₂ SO ₄	460	18	0.039	45	1.8
β-CD-eluate	0.2	7	35.00	18	1600.0

to starting buffer, and the sample was applied to the Mono P column again. Rechromatofocusing of the main forms (forms 4 and 7) yielded a pattern of isomers that was identical with that of the initial chromatofocusing (Figures 9A and 9B). When two of the minor forms (forms 2 and 8) were subjected to rechromatofocusing, a small amount of the particular form remained stable, but the major part was converted into the whole pattern of molecular forms (Figures 9C and 9D). Thus it was proved that the main isomeric forms of the recombinant pullulanase at least are completely interconvertible.

To examine the relation of the tertiary structure of the protein and the formation of isomeric forms, the recombinant pullulanase was unfolded stepwise with urea and the degree of unfolding was assessed by following fluorescence emission and catalytic activity of the pullulanase (Figure 10A). At 1.5 M urea, a transition in fluorescence emission occurred concomitantly with loss of enzyme activity. Obviously the catalytic domain was already affected at this urea concentration, whereas other domains remained folded. At 6 M urea, a second transition occurred, and

Figure 3 Production of recombinant pullulanase RE7 in *E. coli*

BL21(DE3) cells were grown at 27 °C and induced with isopropyl β-D-thiogalactoside at the end of the exponential phase. Cells were harvested 4 h after induction and RE7 was purified as described in the Experimental section; the pullulanase from spinach leaves was purified as described in Ludwig et al. [1]. Samples were separated on an SDS/10% polyacrylamide gel and stained with silver nitrate; lane 1, pullulanase purified from spinach leaves; lane 2, crude extract of *E. coli* BL21(DE3) cells expressing RE7; lane 3, RE7 purified by affinity chromatography on β-cyclodextrin-Sepharose.

Table 3 Substrate specificities and kinetic properties of RE7

Substrate specificities, K_m and K_i , were measured as described by Ludwig et al. [1].

	Pullulanase	
	RE7 (<i>E. coli</i>)	Spinach
Relative activity with		
Pullulan	100	100
Soluble starch	35	37
Amylopectin	16	18
Amylose	0	0
Glycogen	0	0
K_m (app) K_i values		
Pullulan		
K_m (app) (mg/ml)	0.70	0.78
V_{max} (units/mg of protein)	62	58
Amylopectin		
K_m (app) (mg/ml)	7.2	7.0
V_{max} (units/mg of protein)	16	19
β-Cyclodextrin		
K_i (mg/ml)	1.1	0.90

at 10 M urea the pullulanase was completely unfolded. At a concentration of 10 M urea, isoelectric focusing resulted in a single band with a pI of about 5.9 (Figures 7 (lane 3) and 10B).

Analysis of potential post-translational modifications of the pullulanase

The microheterogeneity of the recombinant pullulanase was confirmed by the fact that the described polymorphism was attributable to a single gene product. It was of interest to know however, whether this polymorphism is the result of post-translational modifications of the mature protein. Partial proteolysis of the enzyme could be excluded by the results of N-terminal sequencing and MS (Figure 4). Potential glycosylation of the protein was examined by glycan staining of the blotted

Figure 4 MALDI-TOF-MS of purified spinach pullulanase

(A) Pullulanase from spinach leaves; (B) RE7 from transformed *E. coli* cells. MALDI-TOF-MS was performed as described in the Experimental section. Sinapinic acid was used as a matrix; and calibration was carried out with BSA as standard.

pullulanase from spinach. A 50 μ g sample of pullulanase was not stained whereas 1 μ g of the control protein transferrin was. Thus, taking into account the sensitivity of the glycan assay (25 ng can be detected), glycosylation can be ruled out as a post-translational modification.

Employing gel filtration, Ludwig et al. [1] characterized the pullulanase as a monomeric protein. Changing the pH from 7.0 to between 4.7 and 5.2 resulted in the same retention and elution profile of the pullulanase, which excludes the possibility that protein aggregation is the reason for the existence of the isomeric forms. Ion binding as a potential cause of the formation of the charge isomers was excluded by treatment of the protein with EDTA and subsequent chromatofocusing. No change in the elution profile was observed.

Finally, potential phosphorylation of the pullulanase was investigated by *in vivo* labelling of phosphoproteins in the transformed *E. coli* BL21(DE3) growing on 32 P-containing

medium. In the crude extract, 32 P-containing proteins were detected by autoradiography. The purified pullulanase, however, did not give any signal on X-ray films. Thus it was shown that the recombinant pullulanase is not phosphorylated, and the polymorphism therefore cannot be explained by different phosphorylation states of the protein.

DISCUSSION

A confirmed model for the microheterogeneity of spinach pullulanase

Henker et al. [3] suggested a model for the microheterogeneity of spinach pullulanase in which the phenomenon was attributed to the coexistence of several conformational isoforms of the protein. This model was based on the extensive interconversion of the substrate-induced forms as well as of the individual molecular forms. However, any direct evidence that these isoforms represent

Figure 5 CD spectra of purified pullulanase

(A) Recombinant pullulanase RE7; (B) pullulanase purified from spinach leaves. See the Experimental section for conditions of CD spectrometry.

Table 4 Secondary-structure elements of spinach pullulanase

The values were calculated by comparing the CD spectra of the pullulanase with those of 33 proteins of known structure by the program variable selection [27,28]. Results are means $\pm$ S.E.M.

Structural element	Content (%)
α -Helix	14 $\pm$ 0
β -Sheet	
Antiparallel	28 $\pm$ 1
Parallel	6 $\pm$ 1
β -Turn	17 $\pm$ 0
Others	28 $\pm$ 2
Total	94

a single gene product was lacking, and the possibility that post-translational modifications of the protein were involved in the polymorphism could not be completely excluded.

In the present work the heterologous expression of the spinach pullulanase cDNA in *E. coli* was used to answer these questions. The following findings corroborate the identity of the recombinant and the original protein: both had the same physical and chemical properties, identical N-terminal amino acid sequences, MALDI-TOF-mass spectra, and CD spectra.

Post-translational modifications, such as glycosylation, phosphorylation and protein aggregation, as well as ion-binding phenomena can be excluded as mechanisms creating the micro-

Figure 6 Formation of substrate-induced forms of RE7

Electrophoresis was performed in amylopectin-containing gels under non-denaturing conditions. For each case, 8 μ g of purified pullulanase was applied. II and III indicate the substrate-induced forms II and III, respectively.

Figure 7 Formation of molecular forms of native RE7

Isoelectric focusing of purified pullulanase was performed in the range between pH 4 and 6; protein was visualized by silver nitrate staining. Lane 1, native pullulanase from spinach leaves; lane 2, native pullulanase RE7 from transformed *E. coli*; lane 3, isoelectric focusing of denatured RE7 protein in the presence of 10 M urea.

heterogeneity of the protein. Other modifications, for instance deamidation and methylation, have not been examined. However, the interconvertibility of individual isoforms contradicts such an explanation.

The functional expression of the spinach pullulanase cDNA in *E. coli* showed that translation of a single mRNA can indeed produce the complete spectrum of isoforms. Although the pattern of substrate-induced forms was identical for the recombinant protein and the pullulanase purified from spinach leaves, there were slight differences in the proportions of the molecular forms. These might be attributed to varying oxidation states of the proteins, caused by differing redox conditions in *E. coli* cells and spinach chloroplasts or during the purification procedures. This is indicated by the observation that GSH does not alter the molecular-form distribution of the recombinant pullulanase, but results in a changed pattern of the spinach protein, which is then similar to that of the *E. coli* enzyme (I. Schindler, F. X. Schmid and E. Beck, unpublished work). However, as both proteins can be activated by dithiothreitol, it can be concluded that none of the proteins is in a fully reduced state.

Figure 8 Separation of RE7 isomeric forms

Chromatofocusing on a Mono P column was performed between pH 5.2 and 4.7 as described in the Experimental section. The isomeric forms are designated 1–9.

Figure 9 Interconvertibility of RE7 isomeric forms

Rechromatofocusing experiments were performed on individual fractions shown in Figure 8. (A) Rechromatofocusing of form 4 (fractions 22 and 23); (B) rechromatofocusing of form 7 (fractions 31 and 32); (C) rechromatofocusing of form 2 (fraction 17); (D) rechromatofocusing of form 8 (fractions 36 and 37).

Figure 10 Urea-induced denaturation of RE7

Fluorescence emission and enzyme activity were measured after incubation of the protein for 16 h at different urea concentrations. IEF, isoelectric focusing.

Since under completely denaturing conditions no polymorphism was observed, the formation of a series of isomeric forms is dependent on the native structure of the protein. This result confirms the hypothesis that the isomers represent different tertiary structures of a single polypeptide which coexist under physiological conditions. The shifted pI of the unfolded protein (Figure 10) may be an artifact caused by the high urea concentration [29]. Furthermore the complete interconvertibility of single forms of the recombinant pullulanase into the whole spectrum of isoforms implies that the polymorphism of the enzyme is based on the coexistence of different molecular forms of the protein.

Up until now we have not been able to define the structural elements responsible for the microheterogeneity of the spinach pullulanase. However, the extraordinarily long loop 2 of the catalytic domain as well as domain F, which is specific for branching and debranching enzymes, may be candidates. Deletion studies and site-specific mutagenesis of cysteine residues should provide more information.

The question of whether the described microheterogeneity is unique to pullulanase from spinach leaves or is a more general phenomenon is difficult to answer, because so far no pullulanases from other plants have been examined in this respect. On non-denaturing PAGE, the purified pullulanase from *Bacillus stearothermophilus* separates into several forms that exhibit pullulanase activity [30]. A similar polymorphism has been described for the debranching enzyme from *Thermoanaerobium*

brockii [31] and the α -amylase-pullulanase from *Clostridium thermohydrosulfuricum* [32]. As both enzymes were cloned and expressed in *E. coli*, the described polymorphism cannot be caused by several gene products. Apparently, bacterial pullulanases display a similar polymorphism to that of spinach pullulanase.

The existence of differently charged isoforms of a single protein was also observed by Chen et al. [33] for rabbit muscle creatine kinase. Expression of the cloned gene in *E. coli* yielded the same polymorphism as the protein purified from animal tissue. However, interconvertibility was not examined for this protein. Nevertheless, this example and the case of rabbit muscle adenylate kinase [34] quoted in the preceding paper [3] indicate that protein microheterogeneity, as described for the pullulanase from spinach leaves, is not a unique phenomenon, but may be more widely distributed.

Some aspects of the biological function of the microheterogeneity of spinach pullulanase have been discussed by Henker et al. [3]. The pH-dependent activation achieved by chemical reduction of the protein is due to a shift of the substrate-induced forms in favour of the most active form III. Thus the existence of different substrate-induced forms is a prerequisite for the regulation of pullulanase activity by reduction and oxidation. However, additional modes of activation and inactivation of this form by reduction and oxidation respectively cannot be ruled out. In the absence of substrate, the microheterogeneity of the enzyme becomes apparent via multiple isomeric forms. Since these differ with respect to their specific activities (Figure 8), it could be concluded that the more active isomeric forms are preferentially transformed into the more active substrate-induced form when amylopectin is added as a substrate. Experiments on this are now in progress.

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