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The cDNA sequence of a neutral horseradish peroxidase

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A cDNA clone encoding a horseradish (*Armoracia rusticana*) peroxidase has been isolated and characterized. The cDNA contains 1378 nucleotides excluding the poly(A) tail and the deduced protein contains 327 amino acids which includes a 28 amino acid leader sequence. The predicted amino acid sequence is nine amino acids shorter than the major isoenzyme belonging to the horseradish peroxidase C group (HRP-C) and the sequence shows 53.7% identity with this isoenzyme. The described clone encodes nine cysteines of which eight correspond well with the cysteines found in HRP-C. Five potential N-glycosylation sites with the general sequence Asn-X-Thr/Ser are present in the deduced sequence. Compared to the earlier described HRP-C this is three glycosylation sites less. The shorter sequence and fewer N-glycosylation sites give the native isoenzyme a molecular weight of several thousands less than the horseradish peroxidase C isoenzymes. Comparison with the net charge value of HRP-C indicates that the described cDNA clone encodes a peroxidase which has either the same or a slightly less basic *pI* value, depending on whether the encoded protein is N-terminally blocked or not. This excludes the possibility that HRP-n could belong to either the HRP-A, -D or -E groups. The low sequence identity (53.7%) with HRP-C indicates that the described clone does not belong to the HRP-C isoenzyme group and comparison of the total amino acid composition with the HRP-B group does not place the described clone within this isoenzyme group. Our conclusion is that the described cDNA clone encodes a neutral horseradish peroxidase which belongs to a new, not earlier described, horseradish peroxidase group.

Introduction

Peroxidases catalyse the oxidation of a variety of reductants in the presence of hydrogen peroxide and are found in plants, animals and microbes. Horseradish (*Armoracia rusticana*) peroxidase (EC 1.11.1.7) is one of the most studied peroxidases and is commonly used as a tracer in enzyme immunoassays, histological chemistry and in Southern blots. In terms of its physical properties, the horseradish peroxidase (HRP) is divided into at least five groups of isoenzymes [1,2]. The main isoenzyme groups are: HRP-A, acidic peroxidases with a high carbohydrate content and *pI* around 4; HRP-B, -C, neutral and slightly basic peroxidases with a lower carbohydrate content and *pI* 5.75–9.63 [3]; HRP-D, -E, highly basic peroxidases with a low carbohydrate content and *pI* 10.6–> 12 [1,4].

Physiological functions of the different peroxidase groups are not well documented. Tobacco peroxidase

isoenzymes have been shown to participate in lignification, wound healing and in the defense of the plant against pathogens [5]. Similarities in functions of the different HRP peroxidases are expected.

The complete amino acid sequence of the major isoenzyme belonging to the HRP-C group [6] and a turnip peroxidase isoenzyme, TP 7, [7] have been determined. Three cDNA clones encoding HRP-C isoenzymes have been isolated [8] and one of these clones encodes the same amino acid sequence that was determined by Welinder [6]. However, the cDNA clone encodes 15 more amino acids at the C-terminal end than the amino acid sequenced HRP-C. In this paper, we describe a cDNA clone, isolated from horseradish roots at an early stage of growth, encoding a horseradish peroxidase which is not identical to the earlier described HRP-C.

Materials and Methods

Horseradish

Horseradish roots were obtained from 'Norra Halands Pepparrotsförening', Fjärås, Sweden and cultivated in standard soil in a greenhouse. Fresh sideroots were harvested after 1, 2, 3 and 4 months.

Abbreviation: HRP, horseradish peroxidase.

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A crude preparation of horseradish peroxidase was obtained by the following method. Roots were ground in N_2 [1], homogenized in 0.1 M K_2HPO_4 (pH 9.2) and precipitated twice with ammonium sulfate at 35% and 90% saturation, respectively. The pellet was resuspended in Tris-HCl buffer (pH 7.0) and peroxidase activity was determined [9] and related to the total amount of protein (Bio-Rad Protein Assay, Bio-Rad, Richmond, CA, U.S.A.). Roots with the highest specific activity were selected for the RNA preparation.

RNA extraction

Total RNA was extracted from 10 g of fresh side roots by the method of Cathala [10]. The poly(A) rich mRNA was selected on messenger affinity paper (Hybond-mAP, Amersham International, Amersham, U.K.).

Enzymes

*Eco*RI, *Sac*I and *Xba*I were provided from International Biotechnologies (IBI), New Haven, CT, U.S.A. T4 DNA ligase was provided from Bethesda Research Laboratories (BRL), Gaithersburg, MD, U.S.A.

Construction of cDNA library

cDNA was synthesized from the poly(A) rich mRNA by the Amersham's cDNA Synthesis system. The cDNA was then cloned into a λ gt11 vector [11].

The oligonucleotide probe

A 21 base oligonucleotide probe was chosen on basis of the known amino acid sequence of HRP-C, described by Welinder [6].

The oligonucleotide corresponds to His-40 to Val-46. These amino acids constitute part of a well conserved region around the active site which is similar in many plant peroxidases [5,7,12]. The oligonucleotide was synthesized as a mixture consisting of 64 oligonucleotides differing in the third base of each codon; 3'-GTA/G, AAA/G, GTA/G, CTA/G, ACA/G, AAA/G, CAA-5'. Labeling of the oligonucleotide was made at the 5' end with [γ - ^{32} P]ATP (3000 Ci/mmol, code PB.10168, Amersham International, Amersham, U.K.) and a 5'-end-all biosystems kit (IBI, New Haven, CT, U.S.A.).

Antibodies

The cDNA library was screened with rabbit anti-HRP immunoglobulin, (code B 144, DAKOPATTS, Copenhagen, Denmark) and peroxidase conjugated swine anti-rabbit immunoglobulin (code P 217, DAKOPATTS, Copenhagen, Denmark) was used as the secondary antibody. To remove any antibodies against *Escherichia coli* proteins, the antibodies were, prior to use, exposed to a *E. coli* lysate coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) according to Kohn and Wilchek [13].

Screening of cDNA library

10^5 recombinant phages were screened both with antibodies and the oligonucleotide probe. Phages were induced for expression and replicas for immunoscreening were made on nitrocellulose filters [11]. The phage plates were placed in $+4^\circ\text{C}$ overnight and plaques were then transferred to Colony/Plaque Screen membranes (cat. No. NEF-978) by the method described by New England Nuclear Research Products, Du Pont, Boston, MA, U.S.A.

The immunoscreening was performed as follows. Filters were incubated with primary antibodies for 2 h and then with secondary antibodies for another 2 h, both at 20°C . Antibody binding was detected with H_2O_2 and diaminobenzidine (DAB).

The oligonucleotide screening was proceeded by the following method. Filters were prehybridized at 35°C for 5 h in $5\times$ Denhardt's solution ($1\times$ Denhardt's solution: 0.1 g Ficoll 400, 0.1 g poly(vinylpyrrolidone) M_r 40000 and 0.1 g bovine serum albumin (BSA) in 500 ml H_2O), $5\times$ SSPE ($20\times$ SSPE: 174 g NaCl, 27.6 g $NaH_2PO_4 \cdot H_2O$ and 7.4 g EDTA in 1000 ml H_2O (pH 7.0)), 1% sodium dodecyl sulfate (SDS), 10% dextrane-sulfate and 100 $\mu\text{g/ml}$ heat denatured salmon DNA. The hybridization was then performed in a solution similar to the one described above except that the concentration of Denhardt's solution was $10\times$. The ^{32}P -labelled probe was added to a concentration of 10^6 cpm/ml and the filters were incubated overnight at 35°C . The filters were washed for 5 min and four times with $6\times$ SSC ($20\times$ SSC: 175.3 g NaCl and 88.2 g sodium citrate in 1000 ml H_2O (pH 7.0)) and 0.5% SDS at 20°C and the stringency wash was carried out in $6\times$ SSC and 0.5% SDS at 40°C for 2 min. The filters were then rinsed in $6\times$ SSC at 20°C and autoradiographed overnight (Kodak T-Mat, Eastman Kodak Company, Rochester, NY, U.S.A.).

DNA was extracted from the positive clones [11], separated on agarose gels and transferred to GeneScreen membranes (code NEF-983, New England Nuclear Research products, Du Pont, Boston, MA, U.S.A.). Du Pont's recommendations for transfer were followed. Hybridizations were carried out as described above except that the stringency washes were done at 40° , 50° , 55° , 60° and 65°C .

DNA sequencing

Positive clones were transferred to the pBluescriptII KS+ vector (code 212207, Stratagen, La Jolla, CA, U.S.A.). Both cDNA strands were sequenced from single stranded DNA (ssDNA) with the Sequenase 2.0 and Taq sequence systems (United States Biochemical Corporation, USB, Cleveland, Ohio, U.S.A.). The complete cDNA sequence was obtained by subcloning in *Xba*I and *Sac*I sites which were found at positions +278 and +857 from the 5' end, respectively.

Results

The side roots of horseradish were harvested after 1, 2, 3 and 4 months. Peroxidase activity was assayed and the highest peroxidase activity per total amount of proteins was obtained in the 2 month old side roots. Poly(A)⁺ RNA was isolated from the sideroots at this stage of growth and a cDNA library consisting of 700 000 recombinant phages was then constructed in λ gt11. The cDNA library was screened with a 21-mer mixed oligonucleotide probe based on the amino acid sequence around the distal histidine at the active site of horseradish peroxidase C [6,12,14] and with a polyclonal antiserum against horseradish peroxidase. One oligonucleotide probe positive and two antibody positive clones were isolated after screening 100 000 recombinant phases. The oligonucleotide probe positive clone contained an insert of 1350 bp, while both the two antibody positive clones contained a 1500 bp insert. Hybridization between the 1350 bp insert and the ³²P-labelled 21-mer oligonucleotide probe could be obtained at high stringency conditions (60°C washing temperature) and the probe could be washed away by increasing the washing temperature to 65°C. This

accords well with the theoretically calculated T_m value of 62°C for a perfect match [15]. However, even under conditions where hybridization with the vector could be seen (40°C), none or very weak hybridization could be obtained with the two antibody positive insets. This unspecifically hybridized probe was easily washed away at a stringency temperature of 55°C (Fig. 1).

The nucleotide sequence of the oligonucleotide probe positive clone showed a long open reading frame. Fig. 2 shows the complete nucleotide sequence and the deduced amino acid sequence of this clone.

Comparison of the deduced amino acid sequence with other sequenced peroxidases [16] showed a 53.7% identity with a neutral horseradish peroxidase C isoenzyme [6], 53.8% identity with a basic turnip peroxidase isoenzyme, TP 7 [7] and 52.7% identity with an acidic tobacco peroxidase isoenzyme [5].

The predicted size of the cloned peroxidase peptide is 327 amino acids with an M_r of 35 086. From the cDNA sequence it can be predicted that the protein is synthesized as a preprotein with a 28 amino acid signal sequence (Fig. 2) resulting in a mature protein of 299 amino acids and with an M_r of 32 069 before posttranslational modification.

The deduced amino acid sequence shows five potential N-glycosylation sites, Asn-X-Thr/Ser (Fig. 3) and the mature protein contains nine cysteines which makes it possible to form four disulfide bonds. The predicted sequence of the mature protein moiety has a net negative charge of -2 at pH 7.

Discussion

The two antibody positive clones did not hybridize with the oligonucleotide probe against the active site of peroxidase and their sizes were larger than expected from a full length clone of peroxidase. We concluded that the clones must be encoding for other proteins than peroxidase, to which antibodies in the polyclonal antiserum had also been raised. The double screening of the library, using both antibodies and an oligonucleotide probe, made it possible for us to sort out false positive clones at an early stage. Instead of sequencing three clones, only the oligonucleotide positive clone was sequenced.

The deduced amino acid sequence of the oligonucleotide positive clone could easily be aligned with the sequence of HRP-C and TP 7 (Fig. 3). The sequences around the proximal (His-170) and distal histidine (His-42) are well conserved among plant peroxidases [5,7,12] and could also be identified in our clone. From the predicted amino acid sequence, the region around the H₂O₂ substrate channel [12] was also identified. These sequence agreements clearly identify the oligonucleotide positive clone as a clone encoding a horseradish peroxidase.

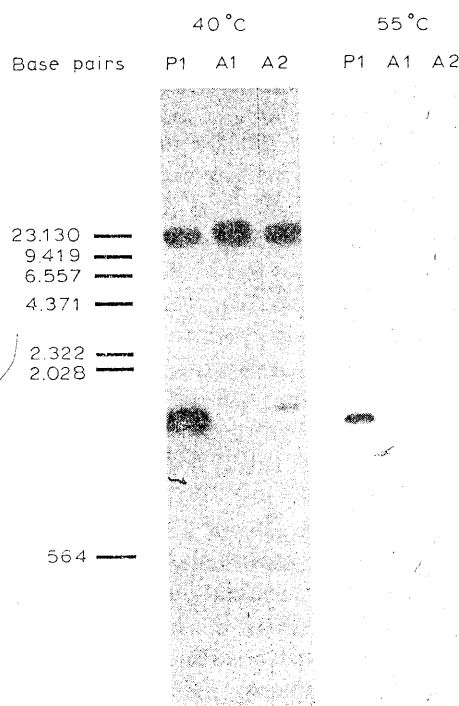


Fig. 1. Southern blots of one probe positive (P1) and two antibody positive clones (A1) and A2) probed with the 21-mer oligonucleotide. Inserts were excised from the positive clones by *Eco*R1 digestion, separated by agarose gel electrophoresis and transferred to GeneScreen membranes. Hybridization was performed with the ³²P-labelled 21-mer oligonucleotide probe and the stringency washes were performed from 40°C to 65°C. Autoradiograms of the 40° and 55°C washes are shown above. Bars to the left indicate the positions of the molecular weight standards.

- 315	TT TTT TTT ACC TTG AAA TAT ATC ATA GAA AAA	
- 252	CCA TAT ACA AAA CAT TAA GAA GAT AAA AAT GGA GAA ATC TGT AAT CAA CAA AAA GCC AAC AAC	
- 189	AAA AGC AAA AGA TGA TCA TTC AAC ACT TAA AAA AAC AAA GCA AAT TTT CCC ACA TCA TAT TAT	
- 126	TCA TAT CTT AAT AAT CAT CTT TAT CTT CTT AAT TAA CCA GAT TCT TTT TCC TTC TTC TCC TCT	
- 63	TCT GTA GCG GTC TTA GGG TGG TAT CCT GGA AGC TTC TCC TTG ATT AAA CAA GCT ATC TAG AAA	
+ 1	ATG AAA ACA CAA ACC AAA GTG ATG GGA GGA CAT GTC TTG CTT ACT GTT TTC ACT CTG TGT ATG <u>Met Lys Thr Gln Thr Lys Val Met Gly Gly His Val Leu Leu Thr Val Phe Thr Leu Cys Met</u>	-8
+ 64	CTT TGT TCA GCG GTT AGG GCA CAG CTA AGC CCT GAC ATT TAT GCT AAA TCG TGC CCG AAT CTT <u>Leu Cys Ser Ala Val Arg Ala Gln Leu Ser Pro Asp Ile Tyr Ala Lys Ser Cys Pro Asn Leu</u>	+14
+ 127	TTA CAA ATT GTC CGT GAC CAA GTT AAG ATC GCC CTG AAG GCC GAG ATA CGG ATG GCT GCT TCT Leu Gln Ile Val Arg Asp Gln Val Lys Ile Ala Leu Lys Ala Glu Ile Arg Met Ala Ala Ser	+35
+ 190	CTC ATT CGT CTT CAT TTT CAC GAC TGT TTT GTT AAT GGG TGT GAT GCG TCT GTA TTG TTG GAT Leu Ile Arg Leu His Phe His Asp Cys Phe Val Asn Gly Cys Asp Ala Ser Val Leu Leu Asp	+56
+ 253	GGA ACC AAC AGT GAG AAA CTC GCG ATC CCA AAC GTG AAC TCT GTG AGA GGA TTT GAA GTA ATT Gly Thr Asn Ser Glu Lys Leu Ala Ile Pro Asn Val Asn Ser Val Arg Gly Phe Glu Val Ile	+77
+ 316	GAT ACT ATC AAA GCC GCT GTG GAA AAC GCA TGT CCT GGT GTT GTT TCT TGT GCT GAT ATA CST Asp Thr Ile Lys Ala Ala Val Glu Asn Ala Cys Pro Gly Val Val Ser Cys Ala Asp Ile Leu	+98
+ 379	ACT CTA GCC GCT CGC GAC TCG GTG TAC TTA AGC GGA GGC CCT CAG TGG AGA GTA GCA TTA GGA Thr Leu Ala Ala Arg Asp Ser Val Tyr Leu Ser Gly Gly Pro Gln Trp Arg Val Ala Leu Gly	+119
+ 442	AGA AAA GAT GGA TTG GTG GCA AAT CAG AGT AGT GCA AAC AAT CTT CCA TCT CCG TTT GAA CCT Arg Lys Asp Gly Leu Val Ala Asn Gln Ser Ser Ala Asn Asn Leu Pro Ser Pro Phe Glu Pro	+140
+ 505	TTA GAC GCT ATT ATT GCC AAA TTT GCA GCC GTA GGC CTT AAC GTC ACC GAT GTC GTA GCT TTA Leu Asp Ala Ile Ile Ala Lys Phe Ala Ala Val Gly Leu Asn Val Thr Asp Val Val Ala Leu	+161
+ 568	TCA GGA GCT CAC ACC TTT GGA CAA GCA AAG TGT GAT CTC TTC AGC AAC CGG CTG TTC AAC TTT Ser Gly Ala His Thr Phe Gly Gln Ala Lys Cys Asp Leu Phe Ser Asn Arg Leu Phe Asn Phe	+182
+ 631	ACC GGC GCG GGA ACT CCG GAC TCA ACA CTT GAG ACA ACA CTT TTG TCT GAT TTG CAA ACA GTT Thr Gly Ala Gly Thr Pro Asp Ser Thr Leu Glu Thr Thr Leu Leu Ser Asp Leu Gln Thr Val	+203
+ 694	TGT CCC ATC GGA GGA AAT GGA AAC AAA ACT GCG CCC CTT GAC AGG AAC TCC ACG GAC GCC TTC Cys Pro Ile Gly Gly Asn Gly Asn Lys Thr Ala Pro Leu Asp Arg Asn Ser Thr Asp Ala Phe	+224
+ 757	GAC AAC AAC TAC TTC AAG AAC CTC CTC GAA GGG AAA GGT CTT TTG AGT TCT GAT CAG ATT CTG Asp Asn Asn Tyr Phe Lys Asn Leu Leu Glu Gly Lys Gly Leu Leu Ser Ser Asp Gln Ile Leu	+245
+ 820	TTC TCG AGT GAC TTG GCA GTG AAC ACA ACA AAG AGA CTA GTG GAG GCT TAT AGT CGG AGC CAA Phe Ser Ser Asp Leu Ala Val Asn Thr Thr Lys Arg Leu Val Glu Ala Tyr Ser Arg Ser Gln	+266
+ 883	TAC TTG TTT TTC AGG GAC TTC ACT TGT TCG ATG ATC AGA ATG GGA AGT CTT GTG AAT GGA GCT Tyr Leu Phe Phe Arg Asp Phe Thr Cys Ser Met Ile Arg Met Gly Ser Leu Val Asn Gly Ala	+287
+ 946	AGT GGG GAG GTT AGG ACA AAC TGC AGG GTT ATT AAT TAA TAA ATT AAT AGT CTG TTT TCG ATT Ser Gly Glu Val Arg Thr Asn Cys Arg Val Ile Asn End	
+1009	TTC TGC AAT GTT TGG GAG TTG ATC AAT CTC TGT GTA TCA AAA TGT TCA CGT GTG TAA TAA TAA	
+1072	GGA TCT ATG GGA TTA TGG GCT TAA AAA AAA AAA AAA AAA AAA	

Fig. 2. The complete nucleotide sequence for the neutral peroxidase cDNA and the deduced amino acid sequence. The amino acid sequence of the predicted translation product is shown below the nucleotide sequence. The HRP coding region is flanked by a 285-bp 5' noncoding region and a 109-bp (exclusive of the poly(A) tail) 3' noncoding region. The initiation and stop codons are marked by bold letters and the apparent signal peptide is underlined. The start of the mature protein begins at position +1. The nucleotide sequence is numbered on the left and the predicted amino acid sequence on the right.

The described clone encodes nine cysteines and one of them is found exclusively in our clone. The position of eight cysteines correspond well with the cysteines found in HRP-C (Fig. 3) and also with cysteines in turnip [7] and tobacco [5] peroxidases. Nine cysteines still give rise to four disulphide bonds and since eight of them are in the same positions as in HRP-C, we can assume a similar three-dimensional structure.

From the deduced amino acid sequence it is predicted that the protein contains a signal sequence of 28 amino acids starting with a few polar residues and continuing with a high content of hydrophobic amino acids. Removal of this signal sequence gives the mature

protein a glutamine as N-terminal amino acid, which can be modified into N-terminal pyrrolidone carboxylic acid, observed in both HRP-C and TP 7 [6,7]. A C-terminal posttranslational modification of HRP-C is also expected after comparing the deduced amino acid sequence from a cDNA clone encoding HRP-C [8] with the determined amino acid sequence [6] of HRP-C (Fig. 3). The amino acid sequence predicted from the cDNA clone described in this paper shows a C-terminal very close to the last cysteine which is conserved among peroxidases, indicating that no posttranslational C-terminal modification occurs with this peroxidase.

The predicted M_r of the mature protein is 32068

Fig. 3. Alignment of the deduced amino acid sequence of the netural peroxidase HRP-n with HRP-C and TP 7. Amino acids are identified by their single letter code. Predicted amino acids corresponding to amino acids in HRP-C and/or TP 7 are underlined. Above the aligned sequence, common plant peroxidase sequence around the proximal and distal histidine is shown and dots indicate the substrate channel [12]. Potential N-glycosylation sites are marked with bold letters. The signal sequence and the C-terminal deduced from a cDNA clone encoding HRP-C are shown in italic. Amino acid sequence adopted: HRP-C, Welinder [6] and TP 7, Mazza and Welinder [7].

which is lower than the M_r calculated from the determined amino acid sequence of HRP-C, molecular weight 33 890. Binding of one mol hemin and 2 mol Ca^{2+} [17] raises the molecular weight to 32 769 and assuming an average carbohydrate composition of two *N*-acetylglucosamine, three mannose, one fucose and one xylose for each of the N-glycosylation sites [14], the molecular weight of the native protein is 38 622. This is significantly lower than the M_r of HRP-C which is 44 000 [14], but is in better agreement with the HRP-E isoenzymes which have lower molecular weights in the range of 34 000–39 000. However, the net charge of the predicted amino acid sequence does not indicate that the isolated cDNA clone encodes a peroxidase of the HRP-E group.

A blocked N-terminal is found in HRP-C, giving the protein a net charge of -2 at pH 7. The calculated net charge at pH 7 of the predicted mature protein of HRP-n is also -2 . However, assuming N-terminal blocking, HRP-n will obtain one negative charge more than HRP-C. These values indicate that the peroxidase described in this paper has either the same or a slightly less basic *pI* value than HRP-C described by Welinder [6]. This excludes the possibility that HRP-n would belong to either the HRP-A, -D or -E groups.

The amino acid sequence comparison showed that

there is not more than 53.7% identity between our clone and HRP-C, and similar identities were found when our protein was compared with turnip and tobacco peroxidases. Therefore, the described cDNA clone encodes a horseradish peroxidase which does not belong to the HRP-C isoenzyme group.

The *pI* value of HRP-n has not been experimentally confirmed and isoenzymes of the HRP-B group have not been cloned or sequenced. However, comparison of the total amino acid composition with the HRP-B group [3] does not indicate that the described cDNA clone encodes a horseradish peroxidase that belongs to this group. Our conclusion is instead that the described cDNA clone encodes a new, not earlier described, horseradish peroxidase group.

The described clone was isolated from a cDNA library prepared from horseradish roots in an early phase of growth. Further studies will be made on the distribution of this isoenzyme during growth of the plant as well as expression of the cDNA clone into an enzymatically active protein.

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