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CELL WALL-BOUND MALATE DEHYDROGENASE FROM HORSERADISH

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Abstract—Isolated cell walls from horseradish contain NAD-specific malate dehydrogenase which is not released on treatment with 2 M NaCl. This enzyme catalyses a rapid reduction of oxalacetate. Its physiological role, however, is assumed to be the oxidation of malate, thus providing NADH as electron donor in the formation of H_2O_2 by a wall-bound peroxidase. In the presence of malate, NAD and Mn^{2+} ions, cell walls catalyse the synthesis of H_2O_2 which might be utilized in lignin formation. In analogy to the known malate–oxalacetate shuttles, the possibility is discussed that this cell wall-associated malate dehydrogenase is involved in the transport of cytoplasmic reducing equivalents through the plasmalemma into the cell wall.

INTRODUCTION

It is well established that the lignin macromolecule is formed by the condensation of hydroxylated cinnamyl alcohols. During the past few years a sequence of enzymes has been detected which catalyse the synthesis of various substituted cinnamic acids and their subsequent reduction to the corresponding alcohols as the immediate lignin precursors (for reviews see [1–4]). Polymerization, the final step in lignin biosynthesis, is initiated by oxidation of these alcohols, and it is now widely accepted that this reaction is catalysed exclusively by a peroxidase (E.C. 1.11.1.7) [5]. This assumption is further strengthened by the well documented existence of cell wall-bound and even cell wall-specific isoperoxidases [6–10]. H_2O_2 required as substrate by these enzymes has recently been detected in high concentrations in xylem and bark of poplar [11]. However, its origin remained obscure (cf. [1–3]). An interesting approach to this question was recently reported by Elstner and Heupel [12]. These authors were able to demonstrate the formation of H_2O_2 by cell walls from horseradish at the expense of NAD(P)H. This complex reaction was ascribed to the activity of a cell wall-bound peroxidase [12] and has also been reported for isolated peroxidases [13, 14]. The problem now arises by which mechanism(s) the NAD(P)H required as electron donor is provided to the bound peroxidase [12]. In this communication, evidence is presented that under the catalysis of malate dehydrogenase (E.C. 1.1.1.37) present in a cell wall-associated form, NADH is produced which is further utilized in the formation of H_2O_2.

RESULTS

Cell wall-bound malate dehydrogenase

Isolated cell walls from horseradish roots were assayed for a variety of NAD or NADP specific oxidoreductases which might eventually provide the reduced pyridine nucleotide required in the formation of H_2O_2. The following enzymes could not be detected (coenzyme used and E.C. number in parentheses): alcohol dehydrogenase (NAD, 1.1.1.1; NADP, 1.1.1.2), glucose-6-phosphate dehydrogenase (NADP, 1.1.1.49), glutamate dehydrogenase (NADH, 1.4.1.2), glyceraldehyde-3-phosphate dehydrogenase (NADH, 1.2.1.9), glyoxylate reductase (NADH, 1.1.1.26) and isocitrate dehydrogenase (NADP, 1.1.1.42). Traces of dehydrogenase activities associated with the cell wall preparations were found for glycerol-3-phosphate dehydrogenase (NADH, 1.1.1.8; 4.1 pkat/mg cell wall), isocitrate dehydrogenase (NAD, 1.1.1.41; 0.7 pkat/mg) and lactate dehydrogenase (NADH, 1.1.1.27; 1.5 pkat/mg).

Appreciable reaction rates were observed only for malate dehydrogenase (NADH, 1.1.1.37) which was by far the most active enzyme (58.2 kpat/mg cell wall). No reaction occurred when NADPH was used as cofactor, or in the absence of either oxalacetate or cell wall preparation. Under the assay conditions described in the experimental section the initial velocity of the reaction was proportional to a maximal cell wall concentration of *ca* 4 mg (1.2 nkat) per ml. Because of the unfavourable equilibrium, the reverse reaction (i.e. oxidation of malate yielding NADH and oxalacetate) could only be demonstrated when an oxalacetate withdrawing auxiliary system (cf. Experimental) was added to the assay mixture. Under these conditions, however, a significant formation of NADH occurred (*ca* 3 nmol/10 min/mg cell wall), and linearity of the reaction with respect to time was maintained for at least 2 hr in the presence of 22.5 mg/ml of cell wall suspension. Apart from the requirement for the above mentioned auxiliary system, the reverse reaction was found to be absolutely dependent on the presence of malate, NAD and cell wall preparation.

Binding-status of malate dehydrogenase

The malate dehydrogenase activity of the cell wall preparations was exclusively associated with the particu-

late material. Furthermore, no enzyme was released under the conditions of the assay even after prolonged incubation periods, thus indicating the existence of strong binding forces between the dehydrogenase and the insoluble cell wall components.

This conclusion was further substantiated when the cell wall preparations were stirred for 2.5 hr at 4° with either 1 M or 2 M NaCl. Only traces (*ca* 1%) of malate dehydrogenase were liberated under these conditions, but the same activity was also found in the supernatant of the water-control. No differences were observed with respect to the wall-bound enzyme activities (364, 386 and 358 pkat/mg cell wall for control, 1 M and 2 M NaCl treated material, respectively).

Attempts to release the wall-associated enzyme by treatment with various enzymes hydrolysing polysaccharides remained unsuccessful. In several of these experiments, the activity of the insoluble material decreased, but no malate dehydrogenase was found in the incubation medium. The occasional detection of this enzyme activity in the ambient liquid in some preliminary experiments must be attributed to microbial contaminations, since no such effects occurred when the incubations were conducted under aseptic conditions.

Malate dehydrogenase and the formation of H_2O_2

The possibility that the NADH formed by this enzyme could serve as an electron donor in the peroxidase catalysed production of H_2O_2 was now investigated. Under the conditions of the malate dehydrogenase assay, formation of H_2O_2 occurred, as determined in the glyoxylate decarboxylation test. As shown in Table 1,

Table 1. Cofactor requirements for H_2O_2 formation by isolated cell walls with malate as electron donor

System	H_2O_2 formation (nmol)	Relative activity (%)
Complete	68.4	100
Minus malate	21.4	27
Minus NAD	22.3	29
Minus Mn^{2+}	26.3	35
Minus oxalacetate withdrawing system	45.1	67

Assay mixtures containing 44 mg cell wall preparation were incubated for 3 hr; for details see Experimental section. Data are corrected for the reaction rate determined in the absence of cell walls.

this reaction depended on the presence of malate, NAD and Mn^{2+} ions; however, an absolute requirement for these cofactors was never observed. This is in accordance with previous results [12] where also some formation of H_2O_2 was observed in the absence of NADH or Mn^{2+}. This might indicate that the cell wall preparations were only partially depleted of these cofactors.

As described above, the formation of NADH required an auxiliary system which influences the reaction equilibrium by withdrawing accumulating oxalacetate. In contrast to this result, H_2O_2 was developed, though at a reduced rate, also in the absence of this auxiliary system (cf. Table 1). This might be due to an analogous effect on the reaction equilibrium, in this instance caused by withdrawal of NADH being utilized in the subsequent synthesis of H_2O_2. The formation of H_2O_2 proceded linearly over a period of at least 6 hr, and the reaction rate was proportional to cell wall concentration up to *ca* 30 mg per assay. The pH-optimum of the reaction was between 7.0 and 7.5.

DISCUSSION

Horseradish cell walls contain a firmly bound malate dehydrogenase which cannot be released by washing with NaCl solutions. This indicates that the enzyme is not attached ionically, but rather exists in a covalently bound form. Thus, it is very unlikely that this wall-bound enzyme represents an artifact resulting from adsorption of cytoplasmic enzyme(s) or residual cytoplasm still present in the preparations. Further support for this interpretation is drawn from the fact that little or no activity was observed of superoxide dismutase and phenol oxidase [12] or of a variety of dehydrogenases known to occur as cytoplasmic, mitochondrial or plastidic components. Similar conclusions were previously drawn when a malate dehydrogenase was released for the first time, among other enzymes, from the walls of potato tubers by a pectate hydrolysing enzyme [15]. In contrast, it has recently been reported [16] that cell walls from tobacco leaf mesophyll are devoid of malate dehydrogenase. This contradictory result, however, may be attributed to the completely different experimental conditions in this investigation.

Concerning the function of the wall-bound malate dehydrogenase, it appears feasible that this enzyme provides the reducing equivalents required in the previously reported formation of H_2O_2 by a cell wall-bound peroxidase [12]. As described, the bound malate dehydrogenase catalyses the formation of sufficient amounts of NADH allowing the production of measurable quantities of H_2O_2, in spite of the presence of an active wall-bound catalase [12]. In this context, it should be mentioned that other enzymes producing H_2O_2 (e.g. glucose oxidase, amino acid oxidase) could not be detected in the cell wall preparations.

The demonstration of a cell wall-bound malate dehydrogenase also demands interest with respect to the following considerations. The reversible oxidation of malate to oxalacetate is regarded to play an essential role in the transport of reducing equivalents through the membranes of chloroplasts or mitochondria (cf. [17–19]). One might visualize such a malate–oxalacetate shuttle across the plasmalemma. By this means, reducing equivalents formed in the cyotplasm would be transported as malate through the outer cell membrane into the cell wall. Subsequent oxidation of malate yields NADH which is utilized in the formation of H_2O_2, whereas the oxalacetate can be reduced again in the cytoplasm. Finally, the H_2O_2 generated by these reactions can function as the oxidizing agent in the formation of phenoxy radicals, which in turn polymerize to the lignin macromolecule.

Such a scheme would offer several advantages. For instance, no transport of toxic H_2O_2 from the cytoplasm to the cell wall is required, since this compound is synthesized directly at the proper site. In this context, it is interesting to note that manganese, required as a cofactor in the peroxidase catalyzed formation of H_2O_2, was detected in sufficiently high concentrations in the wood of sugar maple [20]. Further, the lignification

process could easily be regulated, either by producing H_2O_2 only within the lignifying areas or by destroying H_2O_2 in the non-lignifying parts through the action of a wall-bound catalase which has been found in horseradish cell walls [12].

At present, the proposed scheme is still hypothetical. In spite of the fact that horseradish roots do contain lignin, as shown with the phloroglucinol–HCl stain, it will be interesting to examine whether the reactions described above can also be demonstrated in woody tissues actively synthesizing lignin.

EXPERIMENTAL

Cell wall preparation. Cell walls from horseradish roots were prepared as described in ref. [12]. Comparing the dry wt of the preparations with photometric measurements of dil. suspensions (10 µl/3 ml H_2O) at 500 nm, it was determined that $A_{500} = 1.0$ equals 96 mg/ml dry wt of the original suspension.

Enzyme assays. Cell wall-bound malate dehydrogenase was assayed in reaction mixtures (vol. 1 ml) containing 50 µmol K-Pi buffer, pH 7.6, 0.5 µmol oxalacetate, 0.15 µmol NADH and *ca* 4 mg cell walls. The samples were incubated at 25° with shaking in Warburg flasks for 2 min. Reactions were terminated by rapid suction through Whatman GF/C filter disks. Finally, the concn of residual NADH in the filtrate was determined photometrically. The reverse reaction (i.e. formation of NADH) was determined in assay mixtures (vol. 1 ml) containing 50 µmol K-Pi buffer, pH 7.6, 10 µmol D,L-malate, 5 µmol NAD and *ca* 20–40 mg cell walls, which were supplemented with an oxalacetate withdrawing auxiliary system (10 µmol acetylphosphate, 1 µmol CoA, 10 U phosphotransacetylase, 10 U citrate synthase) and processed as described above. Common standard reaction mixtures were employed in the determination of all other wall-bound enzyme activities, using the above described filtering technique. H_2O_2 formation was measured by a modified procedure according to refs [12, 21]. Assay mixtures (vol. 1 ml) containing the reactants for the reverse reaction (see above) were supplemented with 0.2 µmol $MnCl_2$ and 10 µmol (0.5 µCi) glyoxylate-[1-^{14}C] and incubated with shaking in Warburg flasks at 25°. Reactions were terminated by adding 0.5 ml N H_2SO_4 from the side-arm into the assay mixture. Radioactive CO_2 derived from glyoxylate was trapped in the centre well with 0.2 ml phenethylamine by shaking for 15 min. Aliquots (0.1 ml) were counted in 5 ml of Bray's soln [22].

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