Pyranosone dehydratase from the basidiomycete *Phanerochaete chrysosporium*: improved purification, and identification of 6-deoxy-D-glucosone and D-xylosone reaction products

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Abstract. Pyranose oxidase and pyranosone dehydratase (aldos-2-ulose dehydratase), enzymes which convert in coupled reactions D-glucose to β-pyrene cortalcerone, peaked coincidently during idiophasic growth of *Phanerochaete chrysosporium* under agitated conditions. The enzymes were purified from mycelial extracts of the fungus and separated from each other by hydrophobic interaction chromatography on Phenyl-Sepharose and Phenyl-Superose. Two pyranosone dehydratase activity peaks, PD I and PD II, were resolved. The major PD I fraction, consisting about 74% of the total dehydratase activity, was further purified by anion exchange chromatography on Mono Q to yield apparently pure enzyme as judged by SDS-PAGE and gel filtration on Superose 12. Isoelectric focusing indicated microheterogeneity of the protein by the presence of at least five protein bands with pl 5.1, 5.3, PD II had a pl of 5.75. Overall PD I purification was 60.7-fold with 50% yield. The enzyme acted on several osones (glycosuloses), with the preferred substrate being D-glucosone. D-Xylosone and 6-deoxy-D-glucosone were dehydrated at C-3-C-4 to give the corresponding 5-hydroxy-2,3-dioxoalcanals (4-deoxy-2,3-glycosdiuloses), new enzymatically produced sugar derivatives. The latter labile compounds were trapped as diphenylhydrazine or o-phenylenediamine derivatives and spectroscopically identified. The analogous D-glucosone dehydration product did not accumulate due to its further transformation, pH optimum of PD I activity was 6.0 and its pH stability was optimal at pH 7-11. The enzyme was sensitive to Me2+ chelating agents and some heavy metal ions (Hg2+, Cu2+).

Key words: Pyranosone dehydratase — Aldos-2-ulose dehydratase — Pyranose oxidase — Glucosone — Xylosone — *Phanerochaete chrysosporium*

Pyranosone dehydratase (PD) was originally isolated from plasmolyzed mycelial preparations of the basidiomycete *Corticium caeruleum* by Baute et al. in 1985. The authors demonstrated the enzyme to catalyze conversion of D-glucosone (D-arabino-2-hexosulose) to a β-pyrene compound, cortalcerone, and proposed a reaction mechanism in which the pyranose form of D-glucosone undergoes two enzymatic dehydrations to give an unstable, unsaturated intermediate that yields racemic cortalcerone on spontaneous cyclization (Baute et al. 1987) (Fig. 1). Besides D-glucosone, D-xylosone and 1,5-anhydro-D-fructose could also serve as PD substrates. Dehydratase with the same activity on D-glucosone was recently purified from another fungus, *Polyporus obtusus* (Koths et al. 1992). This enzyme had an apparent native Mr of about 200000 and consisted of two identical subunits with Mr's of 95000. Based on kinetic analysis of cortalcerone production, the latter authors drew a biosynthetic pathway to this metabolite, which is essentially consistent with the above scheme by Baute et al., involving the same unsaturated key intermediate (structure 2, Fig. 1) of the first D-glucosone dehydration. They designated the respective enzyme as aldos-2-ulose dehydratase.

In a previous paper (Volc et al. 1991) we reported cortalcerone synthesis in vitro from D-glucosone by pyranose oxidase (POD) and PD purified from the wood degrading fungus *Phanerochaete chrysosporium*. Separation of these enzymes from each other in the final purification step, gel filtration on Superose 6, was incomplete due to overlapping peaks. We now present a new, simple purification protocol for PD providing higher yields of the pure enzyme and enabling separation of its isoforms. A further purpose of this study was characterization of reaction products from the first osone dehydration by PD and verification of the above mentioned hypothetical reaction scheme for PD mode of action. This was performed by preparation, isolation and spectrometric identification of well defined hydrazone and quinoxaline derivatives of the tricarbonyl sugar reaction products obtained from the dehydrations of D-xylosone and 6-deoxy-D-glucosone.
Fig. 1. Proposed mode of action of pyranosone dehydratase. Possible reaction sequence for conversion of D-glucosone (1) to cortalcerone (5) by pyranosone dehydratase (PD) via unsaturated (2) and tricarbonyl (3) sugar intermediates, according to Baute et al. 1987; shortened. The aldehydic group of 5 is mostly hydrated.

Materials and methods

Chemicals

DMAB and MBTH were obtained from Fluka Chemie (Buchs, Switzerland), N,N-diphenylhydrazine from Koch-Light Lab. (Colnbrook, UK), and o-phenylenediamine, 6-deoxy-D-glucose and BisTris from Sigma Chemicals (St. Louis, Mo., USA). All other chemicals were of the highest purity available. D-Glucosone (D-arabino-2-hexosulose), 6-deoxy-D-glucose, D-galactosone, D-allosone and D-xyllose were prepared by C-2 oxidation of corresponding aldoses using POD purified according to the procedure described below (step 1 - hydrophobic interaction), except that the organism used was the basidiomycete Oudemansiella mucida (Schrad. ex Fr.) Hohn., strain CCBAS 428, which does not produce PD. D-Arabinosone was synthesized via D-arabinose phenylosazone (Bayne 1963).

Organism and culture conditions

Phanerochaete chrysosporium Burds, strain K-3 (CCBAS 571) was obtained from the culture collection of basidiomycetes, maintained at the Institute of Microbiology of the Czech. Acad. Sci., Prague. For a description see Johnsrud and Eriksson 1985. Submerged cultivations were performed on glucose-corn steep-based medium as described previously (Vole et al. 1991).

Enzyme assays

PD activity was assayed by spectrophotometric monitoring the increase in absorbance at 265 nm, where the primary intermediate (probably a ketoenol compound) of D-glucosone dehydration to cortalcerone absorbs (Baute et al. 1985). A standard assay mixture contained 100 μmol BisTris, HCl pH 6.0, 200 μmol D-glucose, and dehydratase to be assayed in a total volume of 2 ml. Before assaying, the fresh solution of the lyophilized substrate (200 mM D-glucose in 50 mM BisTris) was equilibrated at 25 °C for at least 2 h. The reaction was initiated by addition of PD and monitored at 265 nm for 1 min against PD blank solution. One unit of enzyme activity was arbitrarily defined as the amount of activity effecting an absorbance change of 1.00/min at 25 °C.

For determining the pH optimum of PD activity and pH stability enzyme assay reaction mixtures were buffered with either 25 mM piperazine – 25 mM BisTris (pH 4 – 7), 25 mM Tris – 25 mM piperazine (pH 7 – 11), or 50 mM Na phosphate (pH 11 – 12.5). The pH was adjusted with HCl or NaOH.

POD activity was determined by spectrophotometric measuring (590 nm) production of hydrogen peroxide using MBTH + DMAB as the chromogen (Volc and Eriksson 1988). Reaction mixtures contained 200 μmol Na phosphate pH 6.5, 100 μmol D-glucose, 5 μmol DMAB, 0.1 μmol MBTH, 85 nkat horseradish peroxidase and a suitable amount of POD.

Protein concentration was determined by the Folin reagent (Hartree 1972) using bovine serum albumin as the standard.

Enzyme production

Specific activities for POD and PD (Units·g⁻¹ mycelium dry wt.) during the course of cultivation were determined in crude extracts prepared as follows: The contents of triplicate flasks collected every other day from 2 to 16 were combined and washed with distilled water. Mycelium samples (2 g wet wt.) were homogenized in 30 ml centrifuge tubes with 7 ml of 50 mM Na phosphate pH 6.5 for 3 min under cooling in an Ultra-Turrax (IKA-Werk, Staufen, Germany) homogenizer (Shaft 10N, full speed). Homogenates were then centrifuged at 15000 × g for 15 min and the supernatant fractions adjusted to 10 ml. Parallel aliquot samples of mycelia were used for determination of dry weight after drying at 105 °C for 4 h.

Purification of pyranosone dehydratase

Preparation of crude extract. A 11-d mycelium was separated from the fermentation liquid by filtration, washed with distilled water, and after removing excess water suspended (70 g) in 150 ml of 0.1 M Na phosphate pH 6.5 containing 0.3 mM DTT and 0.3 mM PMSF. The suspension was supplemented with 14 g of powdered microcrystalline cellulose and the mycelia disintegrated using an Ultra-Turrax homogenizer (Shaft 18KG, full speed) in five 1-min intervals under ice-bath cooling. The homogenate obtained was then centrifuged for 20 min at 10000 × g at 4 °C and the resulting supernatant (186 ml) designated as crude extract. All the following purification procedures were carried out at room temperature except ultrafiltration steps which were made at 4 °C.