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FUNGAL ENZYMIC ACTIVITY DEGRADING 1,4-α-D-GLUCANS TO 1,5-D-ANHYDROFRUCTOSE

MARIE-ANTOINETTE BAUTE, ROBERT BAUTE* and GÉRARD DEFFIEUX

Laboratoire de Cryptogamie et Microbiologie Industrielle, Faculté de Pharmacie (Université de Bordeaux II), Place de la Victoire, 33000 Bordeaux, France

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Key Word Index—*Morchella vulgaris*; *M costata*; fungi, morels; cortalcerone, microthecin; carbohydrates; pyrones; glucosone; 1,5-D-anhydrofructose, bioconversion; 1,4-α-D-glucan, starch, glycogen

Abstract—1,5-D-Anhydrofructose, the precursor of the antibiotic pyrone microthecin in morels and other fungi, is produced in *Morchella vulgaris* by a novel enzymic activity, presumably a lyasic one, which acts specifically on open-chain 1,4-α-D-glucans; it can be prepared enzymatically *in vitro* from starch in 40 to 50% yield.

INTRODUCTION

In the course of our investigations on the production, by fungi, of the unusual antibiotic pyrones cortalcerone and microthecin, we have shown that they arise through enzymatic dehydration of D-glucosone (D-*arabino*-2-hexosulose) and 1,5-D-anhydrofructose (AF), respectively [1] We reported in 1987 the isolation of AF from the morel *Morchella vulgaris* as the first example of its occurrence as a natural product [2], but, almost at the same time (1986), oxidative activity that converts 1,5-D-anhydroglucitol to AF was reported in a strain of *Pseudomonas* sp. [3]. Should AF formation in fungi conform to the same mechanism, there would be a complete analogy between the biosynthesis of microthecin and that of cortalcerone, since the precursor of the latter, D-glucosone, is derived from D-glucose through a similar oxidation reaction (Fig. 1). This well-established bioconversion, which has been the subject of a series of papers and patents, is still being studied in several laboratories.

Our previous observations on the production of AF and its conversion to microthecin in fungi have shown that: (i) as far as we know, AF and microthecin are produced only by Discomycetes [4] and by some Pyrenomycetes (*Microthecium* and *Melanospora* sp. [5]); (ii) in microthecin-producing fungi, such as *Morchella costata*, conversion of AF to microthecin is inhibited by EDTA [2]; (iii) some species, or strains of AF-producing fungi, are unable to convert this sugar to microthecin, even in the absence of EDTA We used such a microthecin-deficient strain of *Morchella vulgaris* to identify AF [2], and subsequently its precursor, as is reported in this paper.

RESULTS

In cortalcerone- or microthecin-producing fungi, the biosynthesis of these pyrones does not take place unless the fungus is subjected to plasmolytic conditions, e.g freezing–thawing To elucidate whether this 'activation' only occurred with the conversion of AF to microthecin, or also with its production from the unknown precursor, we carried out heat-inactivation of the enzymes on live, undamaged mycelia of the microthecin-deficient strain of *M vulgaris*. If present, the thermostable AF will not be destroyed by this treatment.

TLC examination of the aqueous extract showed the absence of the spot of AF, either when the detection reagent was anisaldehyde–sulphuric acid (which gives a blue colour with AF [2]) or an enzyme preparation from *M costata* that converts AF to microthecin, which yields an orange-red colour with phenylhydrazine [2]. That undamaged mycelia do not contain free AF shows that its biogenesis requires the plasmolytic treatment. Unexpectedly, the enzyme reagent detected a non-migrating substance which gave the orange-red colour with phenylhydrazine (although more slowly than AF). Since this colour was not observed on control plates we inferred that this substance was a substrate for the microthecin-producing enzyme system, and, therefore, was likely to be a precursor of AF. That this water-extractable substance did not migrate on the TLC plates with the solvent used was consistent with a polysaccharide, and we anticipated it might be glycogen, which is known to be widely distributed in fungi.

Incubating *in vitro* pure glycogen with an enzyme extract from the above-mentioned strain of *M vulgaris*–or with an extract from *M. costata* in the presence of EDTA–resulted in the production of AF; microthecin was similarly detected when using an extract from *M costata* without EDTA. Moreover, production of AF (or microthecin) was not inhibited when incubation was carried out under nitrogen, which precludes an oxidative mechanism similar to that observed with 1,5-anhydroglucitol in *Pseudomonas*. In addition, aerobic incubation of this compound with an enzyme extract from the AF-producing, microthecin-deficient strain of *M. vulgaris* resulted in no production of AF, as was demonstrated by TLC examination of the mixture.

Similar results were observed with starch, amylose, amylopectin, dextrins, hydrogenated dextrins, malto-

*Author to whom correspondence should be addressed

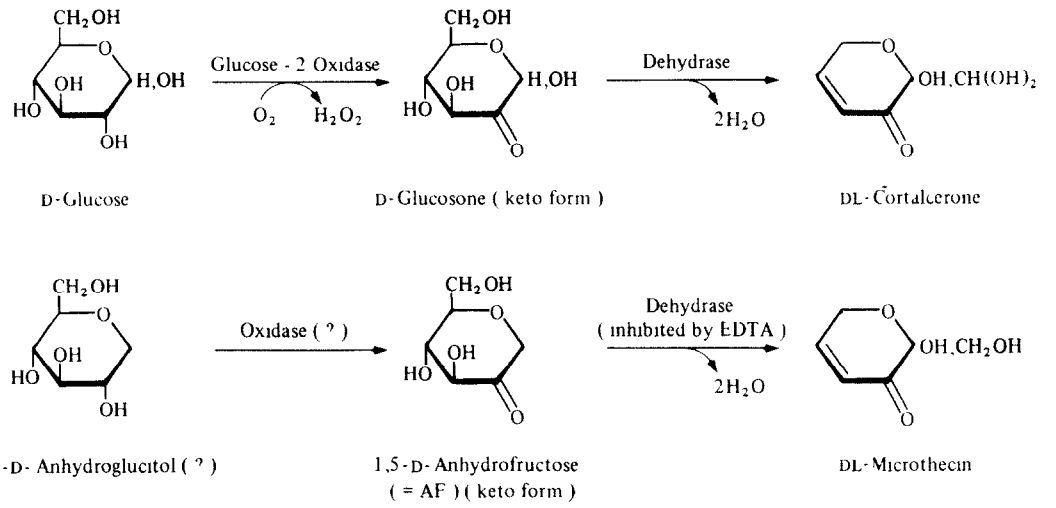

Fig 1 Comparison of the biogenesis of cortalcerone from D-glucose and of microthecin from hypothetical 1,5-D-anhydroglucitol

triose and maltose, on the contrary, neither AF nor microthecin were detected on incubation of the enzyme extract with D-glucose, D-fructose, sucrose, isomaltose, cellobiose, maltitol, dextran, fructosan (inulin), α-cyclodextrin (cyclohexaamylose) and β-limit dextrins. In AF-positive tests, TLC examination of the mixtures also showed the presence of glucose and maltose.

Pure AF could be prepared from starch in 40–50% yield; glucose was removed by baker yeast and maltose by gel filtration, as reported in the Experimental. A residue which did not migrate in the TLC system was identified as β-limit dextrins from its stability to β-amylase

DISCUSSION

Fungi that have been tested possess an enzyme (or an enzyme system) which acts upon open-chain 1,4-α-D-glucans to yield AF (in microthecin-producing strains, AF is subsequently converted to microthecin, unless incubation is carried out in the presence of EDTA), in the fungal cell, the native substrate is likely to be glycogen. Since the reaction is unaffected by anaerobic conditions, biogenesis of AF from α-glucans does not involve an oxidation mechanism That free glucose is not a substrate for the AF-producing enzyme precludes a two-step mechanism involving hydrolytic release of glucose followed by its conversion to AF. *Endo* cleavage of the glucan chains must be excluded, since the enzyme preparation acts neither on cyclodextrins nor on β-limit dextrins Its inactivity on β-limit dextrins and its activity on hydrogenated dextrins also shows that the enzyme does not attack the chain from its reducing end The presence of glucose and maltose among the products of the reaction might result either from minor activity of the enzyme or from its contamination by one or several conventional amylolytic enzymes; this problem will be elucidated only when pure enzyme is obtained

At the present stage of this work, it seems possible to assume that the enzyme (or the enzyme system) would remove successive glucose units from the nonreducing end of the chain and create a double bond between C-1 and C-2, thus yielding the enol form of AF (Fig 2), since

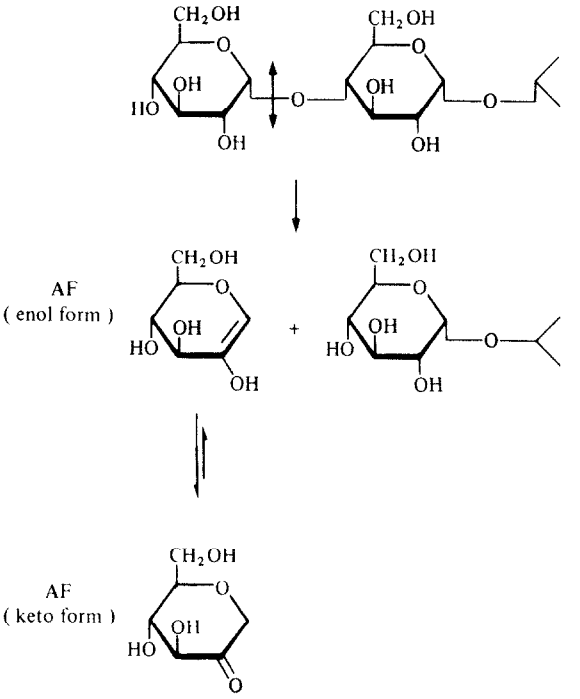

Fig 2 Proposed mechanism for the biogenesis of 1,5-D-anhydrofructose (AF) from 1,4-α-D-glucans

the enzyme does not act upon free glucose, both reactions should be almost simultaneous In branched molecules, the enzyme action is presumably stopped by the presence of 1,6-bonds, which would account for its inactivity on β-limit dextrins and isomaltose, and for the residue of β-limit dextrins. If this mechanism is true, our enzyme would bear some resemblance to the pectine lyases, in so far as those enzymes also yield unsaturated pyranose units The AF-producing enzyme would then be a 1,4-α-glucan lyase

Concerning the biological significance of the production of AF in fungi, its biogenesis from glycogen (or other

1,4-α-glucans) might be an access to the antibiotic pyrone structure of microthecin, which would perhaps give fungi some biological advantage when they are placed in hostile conditions. In species that possess a glucose-2 oxidase, an alternative pathway would be oxidation of glucose to glucosone and conversion of the latter to cortalcerone.

However that may be, the discovery of direct enzymic production of AF from starch and other 1,4-α-glucans offers, as far as we know, the first and unexpected example of an amylolytic activity yielding, as the main product, a compound which is neither glucose nor a structurally related sugar

EXPERIMENTAL

Semi-purified enzyme extract from the microthecin-deficient strain of Morchella vulgaris (*extract 'A'*) was prepared by the same procedure as that from *M costata* (*ext 'B'*) [2].

Aerobic incubation of 1,5-D-*anhydroglucitol.* A soln of 10 mg of 1,5-D-anhydroglucitol in 100 μl of either enzyme ext A or B was incubated at 35° for 2 hr, then analysed by TLC for either AF or microthecin according to previously reported procedures [2].

Heat-inactivation of AF synthesis in the microthecin-deficient strain of Morchella vulgaris. Three 10-day-old mycelia of the fungus (cultivated as previously described [5]) were placed in 150 ml of boiling H_2O, after cooling, they were homogenized in the liquid which was centrifuged for 20 min at 20000 *g*. The supernatant was concd under red. pres to a few ml and analysed for AF by TLC (with pure AF as ref), the anisaldehyde–H_2SO_4 reagent was used for chemical detection, ext B for enzymatic detection [2]

β-Limit dextrins. A suspension of 10 g of sol starch in 100 ml of cold dist H_2O was poured slowly into 300 ml of boiling H_2O. After 1–2 min boiling, the mixt. was cooled to 20° and 25 ml of acetate buffer, pH 4.7 added with 250 mg of β-amylase dissolved in a few ml of dist. H_2O satd with toluene The soln was then allowed to stand at 20° for 24 hr yielding a mixt of β-limit dextrins, maltose and unreacted starch. β-limit dextrins and maltose were sepd from starch by dialysis against H_2O The dialysate (β-limit dextrins + maltose) was evapd to dryness, the residue (2 g) was taken up in 2 ml H_2O and 0.4 ml of this soln was chromatographed on a Fractogel Merck HW 40 (26 × 650 mm column, elution with dist H_2O, 70 ml/hr). β-limit dextrins (20 mg) eluted in the 140–200 ml fraction.

Testing of compounds as possible substrates. Each compound (180 μl of a 20 mg/ml soln) was incubated at 35° for 3 hr with 20 μl of ext A The mixture was then analysed for AF by TLC (detection anisaldehyde).

Anaerobic incubation. A soln of maltodextrin was incubated with the enzyme extract as described above, except that N_2 was bubbled into both solns before they were mixed and then through the reaction mixt. To prevent enzymic oxidation when spotting the liquid onto TLC, the mixt was boiled for 10 min

Preparation of AF from starch. A soln of sol starch (1 g) in 40 ml H_2O containing 40 mg of chloramphenicol (to prevent bacterial contamination) was incubated for 24 hr at 35° with 2 ml of ext A To remove glucose, 400 mg of bakers' yeast were added to the mixt. which was allowed to stand for 24 hr, centrifuged, concd to 2 ml, and introduced onto the top of a 16 mm × 1 m column containing 180 g of Dowex 50 W × 4 (K^+) (200–400) mesh); elution was carried out with dist. H_2O (0.4 ml/min) TLC or HPLC monitoring of the 4 ml-fractions collected showed that AF was contained in fractions 30–40; these were pooled and lyophilized, yielding a white product, *ca* 90% pure. This residue was taken up in 2 ml MeOH and the soln subjected to prep. TLC on silica gel (Kieselgel Merck 60 PF_{254}) with $CHCl_3$–MeOH (7 3); streaks containing AF, which were detected by their white, matt colour or by the triphenyltetrazolium reagent (on a control plate), were combined and eluted by the same solvent. After evapn, the aq. soln of the residue was filtered on a 0.22 μm membrane and lyophilized, yielding 400–500 mg of pure AF.

Residue of β-limit dextrins. The residue of the enzymic reaction was isolated as non-migrating, H_2O-extractable streaks by repetitive prep. TLC (conditions as above), alternating with incubations with enzyme extract A until no more AF was released The aq eluate of the pooled streaks from the ultimate TLC plates was then incubated with β-amylase, as described for the preparation of β-limit dextrins, and the mixt examined by TLC for glucose and maltose.

HPLC. HPLC detection and assay of AF were carried out on a Whatman 25 cm × 4.6 mm Partisil Carbohydrate 10 column; elution MeCN–H_2O (1.5 ml/min); RI monitoring, R_t = 4 4 min

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REFERENCES

1 Baute, R., Baute, M.-A, and Deffieux, G. (1987) *Phytochemistry* **26**, 1395.
2. Deffieux, G, Baute, M.-A., Baute, R, Atfani, M. and Carpy, A (1987) *Phytochemistry* **26**, 1391.
3. Nakamura, T., Naito, A., Takahashi, Y. and Akanuma, H (1986) *J Biochem.* **99**, 605.
4. Baute, M.-A, Baute, R. and Deffieux, G. (1986) *Bull. Soc Pharm Bordeaux* **125**, 12.
5 Baute, M.-A, Deffieux, G and Baute, R (1986) *Phytochemistry* **25**, 1472.