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# The effect of amino acid modifying reagents on the activity of a (1 → 3)-β-glucan synthase from Italian ryegrass (*Lolium multiflorum*) endosperm

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**Abstract**

A series of amino acid modifying reagents were tested for their effects on the activity of the (1 → 3)-β-glucan synthase (EC 2.4.1.34) from endosperm of ryegrass (*Lolium multiflorum*). Of these reagents only the carbodiimide EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), DEPC (diethyl pyrocarbonate), iodine, $Cu^{2+}$, $Hg^{2+}$, phenylmercuric nitrate, *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzylbromide gave significant inhibition. It was concluded that carboxyl and His groups are likely to be essential for enzyme activity but the involvement of Trp residues was not excluded. Significantly, free thiols or disulfides do not appear to be necessary for enzyme activity. The kinetics of the inhibition reaction with diethyl pyrocarbonate were complex. No evidence for methoxyformylation of His was obtained by measurements of absorbance at 242 nm. EDC inhibited in a time and concentration dependent manner, with kinetics suggesting that no reversible complex between the enzyme and the EDC was formed. The apparent bimolecular rate constant was 0.029 $M^{-1}$ $s^{-1}$. The order of the inactivation was 1 indicating that one molecule of EDC is bound per active site. The rate of inhibition is slowed in the presence of UDP–Glc, suggesting that the modified groups may be located in or near the substrate-binding domain. The pH dependence of the rate of inactivation indicates the presence of a critical group with a p$K_a$ of 5.7. These results are consistent with the finding that a 'D,D,D(35)Q(RQ)XRW' motif is present in a number of repetitive β-glycan synthases and suggest that the critical acidic group may be an aspartate. 

*Keywords:* *Lolium multiflorum*; Italian ryegrass; (1 → 3)-β-glucan synthase; Polysaccharide biosynthesis; Amino acid modifying reagents; Carbodiimide; Diethylpyrocarbonate

## 1. Introduction

The (1 → 3)-β-glucan (callose) synthase (EC 2.4.1.34) from the endosperm of Italian ryegrass (*Lolium multiflorum*) grown in suspension culture has been solubilized by detergent extraction from microsomal membranes and enriched by a product entrapment procedure (Henry & Stone, 1982; Bulone, Fincher, & Stone, 1995). The enzyme catalyses the repetitive transfer of glucosyl residues from UDP–Glc in the presence of $Ca^{2+}$ to produce a linear and microfibrillar (1 → 3)-β-glucan with a degree of polymerization of about 1500 (Bulone et al., 1995). The reaction is stimulated by glucosides, especially those bearing hydrophobic groups (Ng, Johnson, & Stone, 1996). The enzyme is competitively inhibited by UDP, one of the reaction products, with an apparent $K_i$ of 200 μM (Meikle, Ng, Johnson, Hoogenraad, & Stone, 1991), and by a photoactivable UDP–Glc analogue, 5-[3-(*p*-azidosalicylamide)]allyl–UDP–Glc ($K_i$ = 16 μM) (Meikle et al., 1991). This analogue irreversibly inhibits the synthase upon UV irradiation (Meikle et al., 1991).

There is no information on the protein domains of the ryegrass (1 → 3)-β-glucan synthase necessary for enzyme activity, and no direct information on the identity of amino acids involved in the catalytic event and very limited data for other β-glycan synthases. This lack of structure-function information is partly a result of the difficulties in purifying membrane-bound glycan synthases to homogeneity, and possibly to diffi-

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Table 1
Effect of amino acid modifying reagents on the activity of *L. multiflorum* (1 → 3)-β-glucan synthase

| Inhibitor tested | Final concentration of inhibitor (mM) | Percent inhibition[a] | Group specificity[b] |
|---|---|---|---|
| Cysteine | 5 | 0 | thiol |
| | 50 | 0 | |
| | 100 | 0 | |
| β-mercaptoethanol | 1 | 0 | thiol |
| | 2 | 0 | |
| | 7 | 0 | |
| *N*-ethylmaleimide | 1 | 0 | thiol |
| | 2.5 | 4 | |
| Methylmercuric iodide | 1 | 9 | thiol |
| | 10 | 24 | |
| Phenylmercuric nitrate | 0.2 | 70 | thiol |
| | 2 | 75 | |
| | 4 | 80 | |
| *N*-acetylimidazole | 25 | 6 | Tyr (amino) |
| Iodoacetamide | 10 | 0 | thiol (Tyr, Met) |
| | 50 | 0 | |
| Cyanate | 20 | 4 | Lys or $NH_2$ |
| | 200 | 11 | |
| | 400 | 20 | |
| Diethylpyrocarbonate (DEPC) | 1 | 11 | His (Tyr, thiol) |
| | 2 | 25 | |
| | 10 | 80 | |
| Iodine | 2 | 2 | Tyr, His |
| | 20 | 12 | |
| $Cu^{2+}$ | 2 | 45 | His |
| | 20 | 69 | |
| | 40 | 86 | |
| $Hg^{2+}$ | 0.2 | 37 | His |
| | 0.5 | 73 | |
| *N*-bromosuccinimide | 5 | 20 | Trp, Tyr (His) |
| | 50 | 40 | |
| 2-hydroxy-5-nitrobenzylbromide | 10 | 70 | Trp (thiol) |
| | 100 | 85 | |
| EDC | 100 | 45 | carboxyl (Tyr, Lys) |
| EDC + glycine methylester | 100 + 1000 | 85 | |
| Woodward's reagent K | 1.1 | 7 | carboxyl |
| | 5.5 | 12 | |

[a]Degree of inhibition expressed as percent of controls without inhibitor.[b]Reactions with residues listed in brackets occur more slowly.

culties in overexpressing these enzymes for which genes or cDNAs are available. Notable exceptions are the chitin synthase 2 of *Saccharomyces cerevisiae* where site-directed mutagenesis has shown the critical role of two Asp residues, and possibly of residues of Gln, Arg and Trp in enzyme activity (Nagahashi et al., 1995), and the expressed cellulose synthase, AcsAB, from *Acetobacter xylinum* where replacement of either $Asp_{189}$ or $Asp_{236}$ by Tyr impaired enzyme activity (Saxena & Brown, 1997).

In this paper, we report the results of a survey of a number of amino acid modifying reagents on the activity of the ryegrass (1 → 3)-β-glucan synthase. We also present a kinetic analysis of the enzyme inhibition by the carbodiimide EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), showing the importance of carboxyl groups in catalysis by the (1 → 3)-β-glucan synthase.

## 2. Results and discussion

### *2.1. Effect of a series of amino acid modifying and other reagents on (1 → 3)-β-glucan synthase activity*

Information on the identity of the amino acid residues that may be essential for (1 → 3)-β-glucan synthase activity was obtained by the use of reagents that react more or less specifically with particular amino acid functional groups. The results are presented in Table 1.

Thiol and disulfide groups are known to be important in a number of ways for the activity of many enzymes. Recently, it was shown that the (1 → 3)-β-glucan synthase from the yeast *Candida albicans* is sensitive to several sulphydryl reactive compounds used in a concentration range of 0–100 μM (Frost, Brandt, Kaufmann, & Goldman, 1995). In the case of the *L. multiflorum* (1 → 3)-β-glucan synthase, however, thiol or disulfide groups do not appear to be necessary for activity. No significant inhibition was observed with cysteine, β-mercaptoethanol, *N*-ethylmaleimide, methylmercuric iodide or iodoacetamide used at concentrations between 1–100 mM. Phenylmercuric nitrate inhibited at concentrations as low as 0.2 mM but it is unlikely that this is due to a reaction with thiol groups since other more reactive thiol reagents did not inhibit. In particular, it would be expected that the small methylmercuric iodide molecule would react more readily with a free thiol and be a more potent inhibitor than would the bulky phenylmercuric nitrate molecule. The reaction of phenylmercuric nitrate with groups other than thiols has been shown to occur with α-amylases (Greenwood & Milne, 1968) and catalase (Sohler, Seibert, Kreke, & Cook, 1952) which are both inhibited by organomercurials, even though thiol groups are either absent or not essential for activity.

Since neither *N*-acetylimidazole nor iodoacetamide significantly inhibited the synthase, Tyr residues do not appear to be essential for activity. Only at quite high concentrations of cyanate (400 mM) was there any inhibition, suggesting that Lys residues are not important for (1 → 3)-β-glucan synthase activity.

DEPC (diethylpyrocarbonate), iodine, $Cu^{2+}$ and $Hg^{2+}$ which react with His residues all gave significant inhibition. The weaker inhibition with *N*-bromosuccinimide could also be due to interaction with His, although involvement of Trp cannot be eliminated. The inhibition due to 2-hydroxy-5-nitrobenzylbromide also suggests a possible involvement of Trp residues in the activity of the (1 → 3)-β-glucan synthase. The carbodiimide, EDC, is a potent inhibitor of (1 → 3)-β-glucan synthase activity, indicating that Glu or Asp carboxyls are important for enzyme activity, although the inhibition observed with Woodward's reagent K (*N*-ethyl-5-phenyloxazolium-3′-sulfonate), which also reacts with carboxyl groups, was much weaker.

In summary, these results show that carboxyl and His residues are most likely to be essential for enzyme activity, although involvement of Trp residues has not been excluded. On this basis, the kinetics of inactivation of the (1 → 3)-β-glucan synthase by DEPC and EDC was studied in more detail.

### *2.2. Inactivation of (1 → 3)-β-glucan synthase by DEPC*

Various concentrations of DEPC were incubated with enzyme for increasing times. The assay mixtures containing radioactive UDP–Glc were then added and enzyme activity measured. The kinetics of inactivation (not shown) were complex. In an attempt to correlate the inactivation with modification of His or Tyr residues, the absorbance of the synthase preparation was followed continuously at 242 and 278 nm over 3 h and after modification with various concentrations of DEPC. The absorbance did not increase at 242 nm during the reaction period, indicating that loss of activity was not concomitant with methoxyformylation of a critical His residue. Nor did the absorbance at 278 nm decrease, suggesting that Tyr residues were not modified (carbethoxylated) by DEPC (Mühlrád, Hegyi, & Tóth, 1967). Cys, Lys and Arg residues are also modified by DEPC (Melchior & Fahrney, 1970) and could contribute to the inactivation, although other reagents that react with these amino acids did not affect enzyme activity.

These results indicate that the inhibition by DEPC that is responsible for the decrease in activity does not result from methoxyformylation of His groups. It is likely that inhibition occurs via a non-specific reaction with amino acid residues that are not necessarily directly involved in catalysis.

### *2.3. Kinetics of inactivation of (1 → 3)-β-glucan synthase by EDC*

Significant inhibition was obtained in the presence of EDC, suggesting that a Glu, Asp or carboxy-terminal amino acid residue might be involved in enzyme catalysis (Table 1). Incubation of the (1 → 3)-β-glucan synthase preparation with EDC resulted in a time- and concentration-dependent inactivation of the enzyme (Fig. 1A). The rate of inactivation as a function of EDC concentration was linear, strongly suggesting that no reversible complex between enzyme and reagent is formed (Fig. 1B). However, it is possible that a reversible complex is formed and that inactivation occurs as illustrated in the following equation (Kitz & Wilson, 1962):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E - I \xrightarrow{k_3} E - X$$

In this case, linear rather than hyperbolic saturation kinetics arise if the rates of dissociation of the complex ($k_2$) and of the formation of the irreversibly inactivated enzyme ($k_3$) are both relatively rapid (Plapp, 1982). The slope of the straight line observed in Fig. 1B gave an apparent bimolecular rate constant of 0.029 $M^{-1}$ $s^{-1}$. The order of the inactivation reaction was deter-

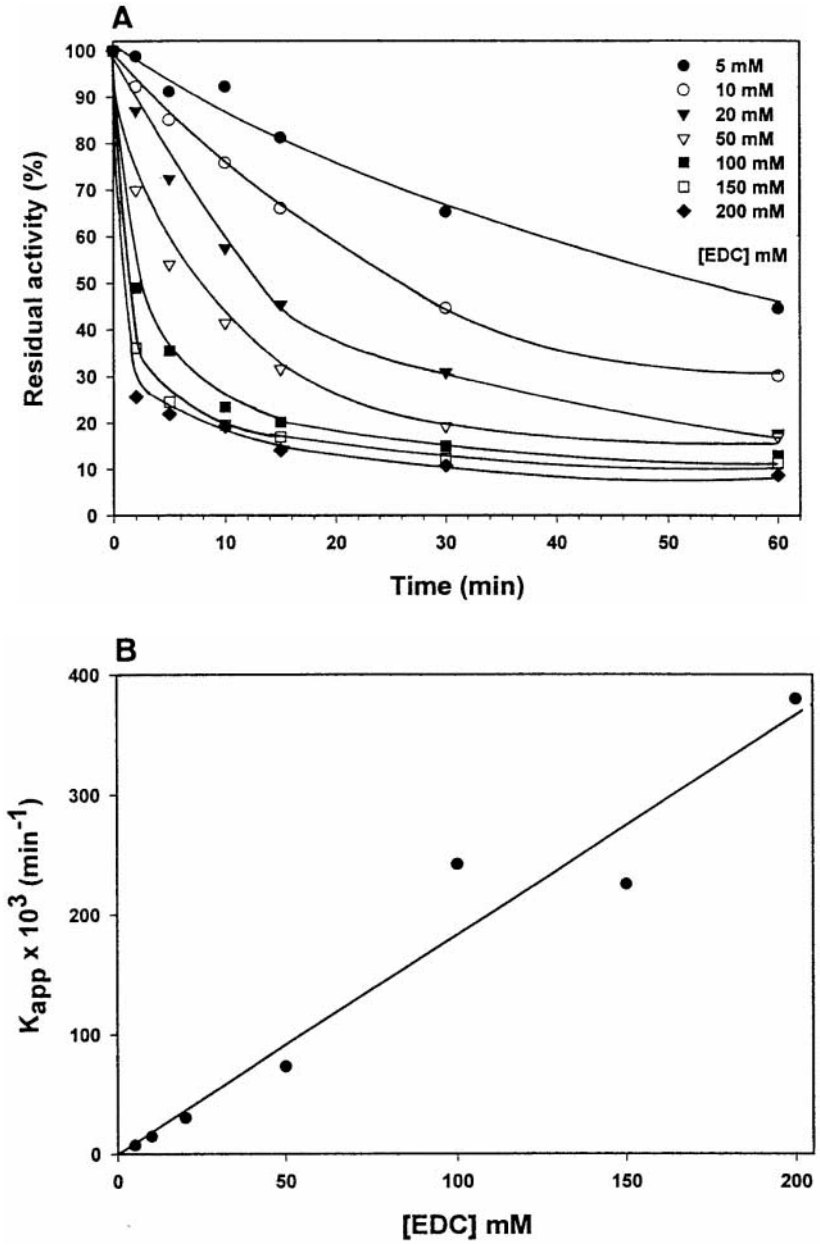


Fig. 1. Inactivation of *L. multiflorum* (1 → 3)-β-glucan synthase by EDC. (A) Time course of inactivation of (1 → 3)-β-glucan synthase by EDC. Residual activity is expressed as percent of controls without EDC. (B) Dependence of rate of inactivation $K_{app}$ on EDC concentration.

mined according to Levy, Leber, & Ryan (1963) from a plot of log $K_{app}$ vs. log[EDC] (Fig. 2). This plot gave a straight line with a slope of 1.07, indicating that the order of the inactivation reaction is 1 and that each inactivated enzyme molecule contains one molecule of bound EDC per active site (Levy et al., 1963).

Modification with 100 mM EDC (without glycine methylester) in the presence of 20 mM UDP–Glc gave a residual activity of 55% after 30 min incubation, compared with 14% residual activity after 15 min incubation in the absence of protecting substrate. This suggests that the modified carboxyl groups may be situated in or near the substrate-binding domain.

The dependence of inactivation rate of *L. multiflorum* (1 → 3)-β-glucan synthase by EDC on pH is shown in Fig. 3. The existence of a critical group for enzyme activity with a p$K_a$ of 5.7 suggests the involvement of the protonated form of either an Asp or Glu residue in the catalytic event. It is now well-established that enzymic glycoside hydrolysis involves an acid–base catalytic cycle of reactions with carboxyl amino acids participating both as acid and base (Koshland, 1953; Sinnott, 1990; Withers & Aebersold, 1995). The formation of glycosidic linkages can formally be regarded as the reversal of hydrolysis. Thus, it is not unexpected that the formation of glycosidic linkages

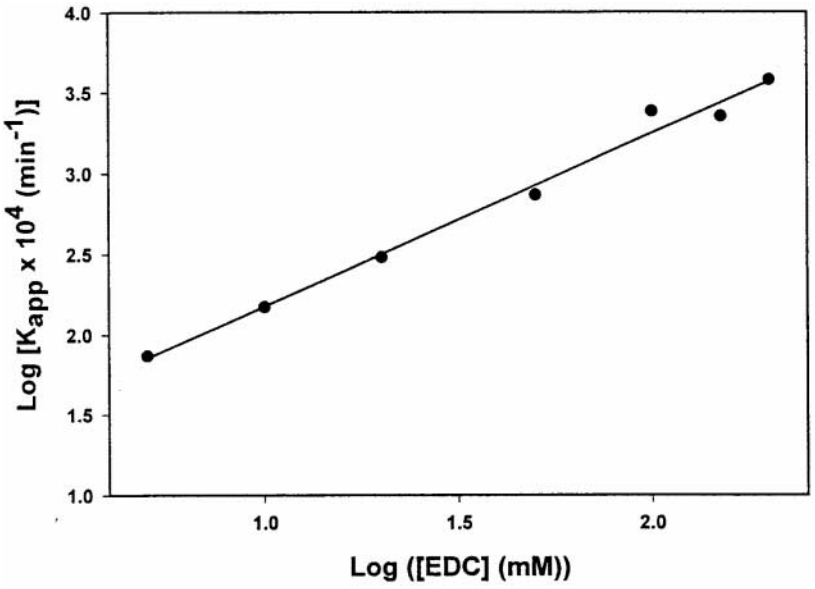


Fig. 2. Determination of the apparent order of the inactivation reaction. The slope of the curve is 1.07.

would also involve participation of carboxyl amino acids. This result lends support to the proposal (Saxena, Brown, Fèvre, Geremia, & Henrissat, 1995) based on hydrophobic cluster analysis of amino acid sequences deduced from genes encoding a number of β-glycosyltransferases, including cellulose and chitin synthases, that all repetitive enzymes in this group contain a 'D,D,D(35)Q(R,Q)XRW' motif, and that the conserved Asp residues are present in the putative active site region. The identical motif is also present in the polypeptide encoded by the gene for a (1 → 3)-β-glucan synthase from an *Agrobacterium* sp. (Stasinopoulos, Fisher, Stone, & Stanisich, 1998). as well as in the deduced polypeptides of β-glycan synthase cDNAs or genes from cotton (*Gossypium hirsutum*) (Pear, Kawagoe, Schreckengost, Delmer, & Stalker, 1996), *Arabidopsis thaliana* (Saxena & Brown, 1997), *Acetobacter xylinum* (Saxena & Brown, 1997), *Agrobacterium tumefaciens* (Matthysse, White, & Lightfoot, 1995), *Streptomyces pyogenes* (Dougherty & van de Rijn, 1994), *Streptococcus pneumoniae* (Arrecubieta, Garcia, & Lopez, 1995; Dillard, Vandersea, & Yother, 1995), *Rhizobium meliloti* (Jacobs, Egelhoff, & Long, 1985) and *S. cerevisiae* (Nagahashi et al., 1995). More direct evidence for the role of carboxyl residues in β-glycan synthases comes from site-directed mutagenesis of chitin synthase 2 from *S. cerevisiae* which shows that two Asp residues

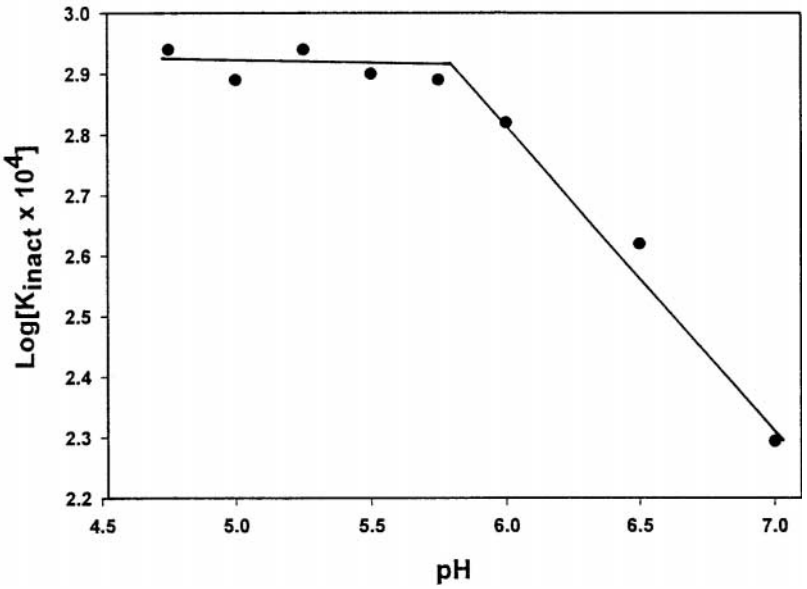


Fig. 3. Dependence of inactivation rate of *L. multiflorum* (1 → 3)-β-glucan synthase by EDC on pH.

are located in the active site and function as catalytic residues of the enzyme, possibly together with non-acidic groups (Arg, Trp and Gln) (Nagahashi et al., 1995), and in AcsAB (Saxena & Brown, 1997) where the replacement of the first or second Asp residue led to impairment of in vitro enzyme activity. It is noteworthy that our inhibition results show the involvement of an acidic amino acid, possibly together with His and Trp residues in catalysis by the ryegrass (1 → 3)-β-glucan synthase, and that, recently, Asp ($Asp_{413}$), Trp ($Trp_{491}$) and His ($His_{561}$) have also been identified by a site-directed mutagenesis approach as essential for the bacterial (*Streptococcus mutans*) GTF-I glucosyltransferase involved in the synthesis of (1 → 3)-α-rich glucans (Tsumori, Minami, & Kuramitsu, 1997).

In summary, our results provide the first direct experimental evidence that an acidic amino acid is involved in the catalytic mechanism of *L. multiflorum* (1 → 3)-β-glucan synthase, although His and Trp residues may also be involved. They support the notion that, by analogy with the reaction mechanism of polysaccharide hydrolases, an acid–base mechanism may be expected in the catalysis of polysaccharide biosynthesis (Sinnott, 1990).

## 3. Experimental

### *3.1. Preparation of microsomal membranes and CHAPS-solubilization of (1 → 3)-β-glucan synthase*

Cells from *Lolium multiflorum* endosperm cultures (Smith & Stone, 1973) were harvested in mid-log phase 8–10 days after inoculation. Microsomal membranes were isolated by differential centrifugation of a cell homogenate made in a French pressure cell and membrane-bound proteins solubilized with 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate) in 100 mM MOPS/NaOH buffer, pH 7.5, containing 10% glycerol and 2 mM EDTA and EGTA (Bulone et al., 1995).

### *3.2. (1 → 3)-β-Glucan synthase assay*

Solubilized (1 → 3)-β-glucan synthase was assayed in a total volume of 400 μl 25 mM MOPS/NaOH buffer (pH 7.5) containing 20 mM cellobiose, 8 mM $CaCl_2$, 1 mM UDP–D-glucose and 0.4 μCi UDP–[$^{14}$C]-D-glucose. The product synthesized in these conditions has clearly been identified as a (1 → 3)-β-glucan (Bulone et al., 1995). Assays were performed in duplicate at 25°C. The radioactive, 66%-ethanol–insoluble (1 → 3)-β-glucan synthesized after 30 min incubation was retained on glass fibre filters and radioactivity measured in 5 ml toluene-based scintillant as previously described (Bulone et al., 1995).

### *3.3. Inhibitor assays*

The effect of amino acid modifying reagents and other compounds on (1 → 3)-β-glucan synthase activity was investigated by incubating the enzyme with each compound (Table 1) and assaying the residual activity. Inhibitors were added to the reaction mixture with the substrate (1 mM UDP–glucose). Inhibitors soluble in organic solvents were prepared as concentrated solutions and added in a small volume (1–2 μl) to the assay mixture. Controls were run in parallel with organic solvents only, and the degree of inhibition was calculated by comparison with the controls.

Kinetic experiments were conducted with DEPC and EDC. CHAPS-extracted enzyme (100 μl) was pre-incubated with DEPC (0, 0.6, 1, 2, 4, 8, 10, 15 and 20 mM final concentrations) or EDC (0, 5, 10, 20, 50, 100, 150 and 200 mM) for various times (0, 2, 5, 10, 15, 30 and 60 min). The (1 → 3)-β-glucan synthase assay mixture was then added to each tube and enzyme activity measured for each condition as described above.

The dependence of (1 → 3)-β-glucan synthase inactivation by EDC as a function of pH was studied in the presence of 20 mM EDC at pHs varying from 4.75–7.0. These pHs were obtained by adding various amounts of 100 mM MES buffer to the enzyme preparation. For each pH the enzyme was pre-incubated with EDC for various times (0, 2, 5, 10, 15 min). The assay mixture containing radioactive substrate was then added to each tube and enzyme activity assayed as described above. The pH of the reaction mixture (7.5) was checked at the end of incubation. Inactivation of enzyme was measured by comparison with a control performed in parallel, in the same conditions (pH and time of incubation), but without inhibitor.

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