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Enzyme kinetics and chemical modification of α-1,4-glucan lyase from *Gracilariopsis* sp.

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Abstract

The kinetic properties and active site amino acids of α-1,4-glucan lyase from the marine red macroalga *Gracilariopsis* sp. were examined. Using ^{1}H NMR spectroscopy the α-1,4-glucan lyase was found to degrade α- and β-maltose at different rates. The effect of pH on the kinetic constants suggested the presence of two catalytically important amino acids in the active site with pK_a values of 3.5 and 6.2. The former indicated the presence of an ionised aspartate or glutamate residue in the active site. This was tested using the carboxyl specific reagent EDAC, which inhibited enzyme activity in a time dependent manner when an external nucleophile was added. No protection against the inactivation was obtained by addition of amylopectin, maltitol or 1-deoxinojirimycin. Inactivation decreased V_{max} over 2.5-fold with little effect on K_m which supports the direct involvement of a carboxyl group in catalysis.

Keywords: *Gracilariopsis* sp.; Gracilariales; Rhodophyta; α-1,4-glucan lyase; Kinetic constants; Chemical modification; EDAC; Carboxyl groups

1. Introduction

The enzyme α-1,4-glucan lyase (EC 4.2.2.13), isolated from the red alga *Gracilariopsis* sp. (Gracilariales, Rhodophyta), was the first enzyme described in a new class of starch degrading enzymes (Yu et al., 1993). It degrades α-1,4-glucans ranging from maltose to amylose and amylopectin by successive removal of glucose units from the non-reducing end as 1,5-anhydro-D-fructose. The product, 1,5-anhydro-D-fructose, has been identified from several different organisms; bacteria (Nakamura et al., 1986), fungi (Baute et al., 1988), rat liver (Kametani et al., 1996) and human cell lines (Suzuki et al., 1996).

However, the α-1,4-glucan lyase has only been isolated from two species of fungi (Yu et al., 1997). The fungal enzymes share the reaction mechanism with the algal lyases but are distinct in several other respects and are thought to represent a separate lyase family. The fungal enzymes also have similarities in their active sites to starch hydrolases but no sequence similarities were found (Yu et al., 1997). The role of the α-1,4-glucan lyase in algal metabolism is unknown but enzyme activity varies greatly, suggesting a physiological importance. In this paper the algal α-1,4-glucan lyase is further characterised regarding kinetic properties and active site amino acids.

2. Results and discussion

2.1. NMR spectroscopy

The degradation of an equilibrated sample of maltose (ca 40% α- and 60% β-maltose) by α-1,4-glucan lyase was followed by ^{1}H NMR to examine if the enzyme has a preference for either form. Different

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reaction orders were observed for the degradation of the two anomers (Fig. 1).

Degradation of β-maltose followed first order kinetics while α-maltose showed apparent zero order reaction kinetics. However, the extent of utilisation of the α- and β-anomers was similar over the time period studied. To our knowledge differences in the reaction orders to the utilisation of α- and β-maltose have not been reported for other glycolytic enzymes and is an interesting feature of the α-1,4-glucan lyase. The effect of the α- or β-configuration of the substrate on enzyme activity is likely to decrease with increasing chain length, but this was not possible to confirm as the peaks from maltose and maltotriose overlapped in ^{1}H NMR spectra.

2.2. K_m and V_{max} values

Although the NMR data clearly indicate different reaction kinetics, no statistical differences were obtained between the K_m and V_{max} values for newly dissolved maltose (96% β-maltose) and those of equilibrated maltose (60% β- and 40% α-maltose) ($p > 0.5$ for both K_m and V_{max}, t-test, $n = 4$). Thus, we found no practical implications of the different degradation rates. The K_m (maltose) was 1.24 ± 0.21 mM and V_{max} 16 ± 2 nmol/min. It is, however, possible that the kinetic constants for α-maltose would be different from those of β-maltose. The K_m value observed for α-1,4-glucan lyases was within the range reported for α-glucosidases from plants (Dey and Campillo, 1984).

2.3. Effects of different carbohydrates

Several different mono- and disaccharides, the majority non-degradable by α-1,4-glucan lyase, were tested as inhibitors; few showed any inhibiting effect. Of the compounds tested only *p*-nitrophenyl α-maltoheptaoside (1 mM), amylopectin (0.48 mg/ml), maltitol (100 mM) and deoxynojirimycin (0.1 μM) resulted in a substantial inhibition of enzyme activity (in the range of 36–53% with maltose as substrate). Among those that were not effective as inhibitors were arabinose, galactose, glycerol, lactose, xylose and D-glucuronic acid (all at 100 mM). The effects of amylopectin, maltitol and deoxynojirimycin on K_m and V_{max} of α-1,4-glucan lyase were investigated in an attempt to determine if the enzyme has two active sites for differently sized substrates (Table 1). This has been reported for several α-glucosidases (Dey and Campillo, 1984) and was also suggested for α-1,4-glucan lyase (Yu et al., 1993). Addition of deoxynojirimycin or maltitol competitively inhibited the enzyme activity toward maltose while addition of amylopectin resulted in mixed inhibition (data not shown). If maltose and amylopectin were degraded at the same site they would be expected to act as competitive inhibitors of each other. Thus, the question of different active sites for maltose and amylopectin remains unresolved.

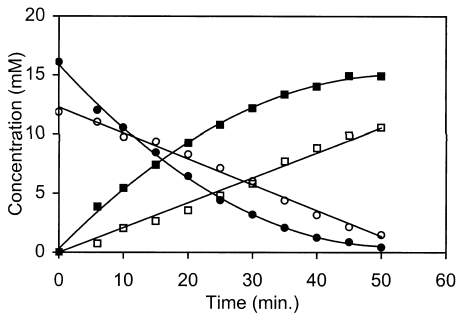

Fig. 1. Degradation of an equilibrated solution of maltose (28 mM) in D_2O by α-1,4-glucan lyase followed by ^{1}H NMR. The concentrations of glucose and maltose in α- and β-configuration were calculated from the values of the integrated peaks (○, α-maltose; ●, β-maltose; □, α-glucose; ■, β-glucose).

2.4. Effect of pH on K_m and V_{max}

The effect of pH on the K_m and V_{max} values were examined (Fig. 2) in order to investigate which amino acids might be present in the active site. Below pH 3 the enzyme kinetic constants could not be determined due to the low activity of the enzyme. The data indicated the presence of two active groups with pK_a values of 3.5 and 6.2. As maltose is uncharged at these pH values the changes in pK_a reflected changes in the properties of the enzyme rather than the substrate. The pK_a of 3.5 is a good indication of an ionised aspartate or glutamate residue. The pK_a value of 6.2 was more difficult to assign but could be compatible with the requirement of a cysteine residue for catalytic activity. This would be in agreement with the observed inactivation by SH-specific reagents. The pH profile of the enzyme may allow regulation by pH changes in the chloroplast stroma as suggested for pea chloroplast α-glucosidase (Sun et al., 1995).

2.5. Effects of different reagents

Effects of various reagents on α-1,4-glucan lyases

Table 1
Inhibitor constants

Inhibitor	*n*	$K_i \pm$ s.d. (against maltose)	$K_i' \pm$ s.d. (against maltose)
Amylopectin	18	0.05 ± 0.02 mg/ml	0.20 ± 0.08 mg/ml
Deoxynojirimycin	4	0.033 ± 0.002 μM	Not applicable
Maltitol	9	23.8 ± 5.2 mM	Not applicable

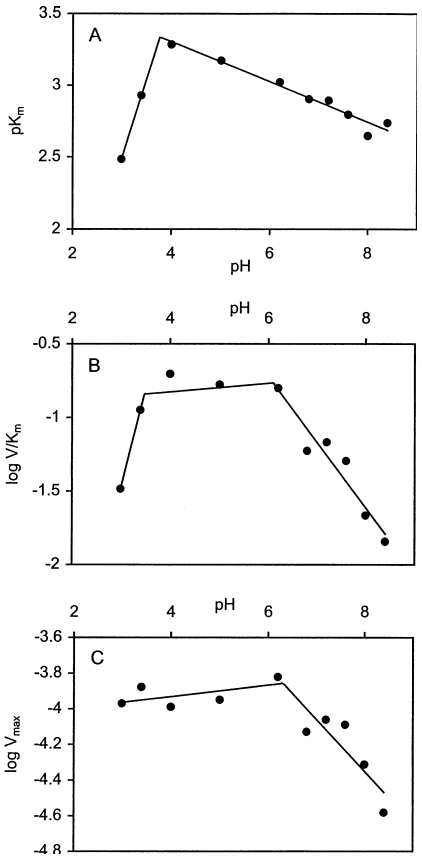

Fig. 2. pH-dependence of the apparent kinetic constants of α-1,4-glucan lyase (maltose). The apparent kinetic parameters were determined by the direct linear plot. (A) pK_m, (B) $\log V_{max}$ and (C) $\log V/K_m$.

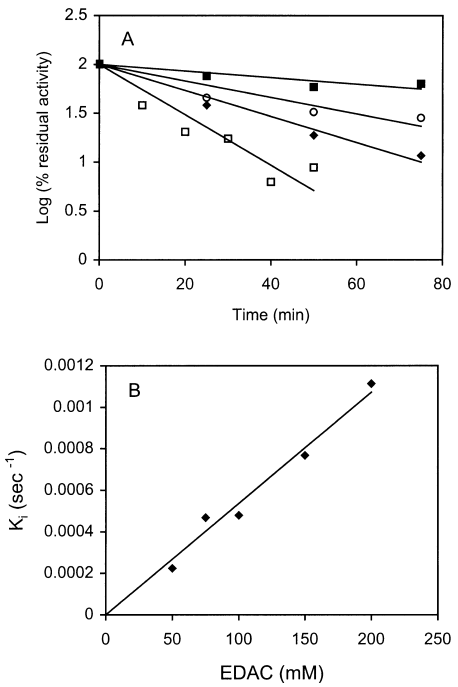

Fig. 3. Inactivation of α-1,4-glucan lyase by EDAC and GEE. (A) The enzyme was modified with various concentrations of EDAC (■, 50 mM; ○, 100 mM; ◆, 150 mM; □, 200 mM) and 250 mM GEE in 200 mM MES–NaOH buffer (pH 4.8) at 30°C. Inactivation was quenched at the indicated times and the residual activity A/A_0 was determined as described in Experimental. (B) Plot of the pseudo-first order inactivation rate constants as a function of the EDAC concentration. The slope represents the second-order inactivation rate constant (0.3 ± 0.08 M^{-1} min^{-1}).

were investigated (Table 2). Reagents such as $HgCl_2$ and PCMB (*p*-chloromercuribenzoic acid), acting on SH-groups, inhibited enzyme activity on both maltose and amylopectin. This indicates that cysteine residues were important for the binding of substrate or in the catalytic activity of the enzyme. However, as no substrate protecting effect was found with either substrate (data not shown) the SH-group(s) are probably not located within the active site of the enzyme but could instead be involved in substrate binding, enzyme motility or be important for the structural integrity of the protein. SH-reagents have also been shown to inhibit α-1,4-glucan lyases from fungi (Yu et al., 1997) and α-glucosidase from rice and grapes (Takahashi and Shimomura, 1973; Peruffo et al., 1978). Pyridoxal-5-phosphate affects lysine groups and had no inhibitory effect. Little or no inhibition was observed with TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone), specific for histidine, and TLCK (*Nα-p*-tosyl-L-lysine chloromethyl ketone), acting on serine groups.

Table 2
Effect of amino acid modifying agents on activity of α-1,4-glucan lyase using maltose and amylopectin as substrates

Modifying agent	mM	% of control maltose	% of control amylopectin	Amino acid group targeted
p-Chloromercuribenzoic acid	2	8	8	Cysteine
$HgCl_2$	10	14	7	Cysteine
Pyridoxal-5-phosphate	1	96	115	Lysine
N-Tosyl-L-phenylalanine chloromethyl ketone	2	56	76	Histidine
Nα-p-Tosyl-L-lysine chloromethyl ketone	2	63	100	Serine

2.6. Effect of EDAC and nucleophiles on α-1,4-glucan lyase activity

The amino acid modifying agent EDAC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide), specific to the carboxyl groups of glutamate and aspartate, was used to examine further the possibility of a carboxyl group in the active site. The α-1,4-glucan lyase was suitable for modification since it is stable at pH 4–5 where EDAC modifies proteins most efficiently. Carboxyl modification by carbodiimides proceeds through the formation of an unstable *O*-acylurea adduct of the protonated carboxy group. The activated carboxyl group can then react by one of the following routes (i) regeneration of the original carboxyl group through hydrolysis, (ii) formation of a stable *N*-acylurea, (iii) reaction with an added or neighbouring nucleophile such as a lysyl ε-amino group (Carraway and Koshland, 1972; Williams and Ibrahim, 1981). An external nucleophile, GEE (glycine ethyl ester), had to be added to the reaction in order to achieve inhibition. In the absence of external nucleophile, the enzyme was fully stable in the presence of EDAC. Incubation with GEE alone had no effect on enzyme activity and increasing the concentration of GEE to 500 mM did not further decrease the activity (not shown). The lack of inhibition unless GEE was added support the direct involvement of the carboxyl group as it shows that the inactivation is not due to internal crosslinking to other amino acids. Carbodiimides can react not only with carboxylic acids, but also with other side groups such as thiols and phenols. However, only in the case of carboxyl groups can nucleophilic reagents modify the rate of chemical inactivation by carbodiimides (Carraway and Koshland, 1972; Williams and Ibrahim, 1981).

The semi-logarithmic plots of residual enzyme activity against EDAC concentration were essentially linear (Fig. 3(A)), indicating that the inactivation followed pseudo first order kinetics. The plot of first order inactivation rate constants (K_i) against EDAC concentrations was linear, allowing the determination of a second order inactivation rate constant of 0.3 ± 0.08 M^{-1} min^{-1} and indicated the occurrence of an essentially irreversible complex between the inactivation agent and the enzyme. A double logarithmic plot of the pseudo first order inactivation rate constant versus the inactivator concentration (Fig. 3(B)) produced a slope of 0.97 ± 0.02 indicating a stoichiometry of the inactivation reaction of one with respect to EDAC. The concentration of EDAC needed to obtain inhibition was relatively high compared to other studies (50–200 mM), however, similar concentrations were used for the modification of gluthathione transferase, 100 mM (Xia et al., 1993) and oxalate oxidase, 150 mM (Kotsira and Clonis, 1998). It could partly be due to high levels of glutamic acid and aspartic acid in the α-1,4-glucan lyase (Yu et al., 1993).

2.7. Effect of EDAC inactivation on K_m and V_{max}

The α-1,4-glucan lyase was partially inactivated by treatment with 75 mM EDAC and 250 mM GEE for 40 min and the apparent K_m and V_{max} toward maltose were determined. Inactivation did not significantly affect the apparent Michaelis constant, 1.61 ± 0.17 mM in the modified enzyme as compared to 1.27 ± 0.14 in the native enzyme. The apparent V_{max} on the other hand decreased to 40% of V_{max} for the unmodified enzyme. This supports a direct role of the carboxy group in catalysis rather than in binding of ligands. In several α-glucosidases aspartic acid (Iwanami et al., 1995) and glutamic acid (Jenkins et al., 1995) have been identified as the catalytic groups. Addition of 10 × apparent K_m or K_i of maltitol, amylopectin or deoxynojirimycin to the inactivation reaction had no effect on the modification of α-1,4-glucan lyase by EDAC.

2.8. Effect of pH on EDAC inactivation

The effect of pH on the inactivation with EDAC was studied in the pH range 4.6–6.0 (Fig. 4). The inactivation was less efficient at higher pH and the pK_a value of the modified group evaluated with Eq. (5) was 2.42 ± 0.06, which is lower than but comparable to the value obtained from the effect of pH on the K_m and V_{max} of the enzyme (pK_a 3.1).

The importance of a carboxy group for the catalytic activity of α-1,4-glucan lyase was established and is a further indication of the active site similarity to α-glucosidases. The results also suggested the presence of a cysteine in the active site.

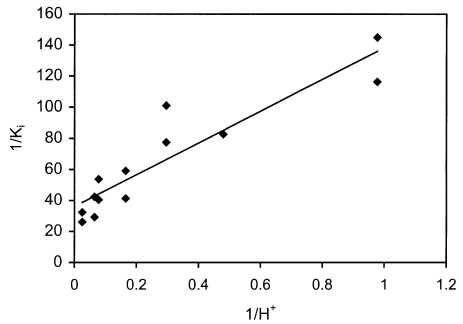

Fig. 4. pH dependence of the inactivation of α-1,4-glucan lyase by EDAC and GEE. The inactivation of the enzyme was performed for 45 min at 30°C with 150 mM EDAC and 250 mM GEE in MES–NaOH buffer at pH 4.5–6.0.

3. Experimental

3.1. Chemicals

1,5-Anhydrofructose, >95% pure as determined by NMR, was produced by incubating soluble starch with α-1,4-glucan lyase followed by purification by gel filtration and was a kind gift from Dr. Anders Broberg. All other chemicals were obtained commercially.

3.2. Enzyme isolation

α-1,4-Glucan lyase was prepared to homogeneity according to Yu et al. (1993) from *Gracilariopsis* sp. (Goff et al., 1994), earlier described as *Gracilariopsis lemaneiformis* (Yu et al., 1993, 1995). The specific activity of the purified enzyme was 960 U/mg. The alga was collected in Zhan Shan Bay, Qingdao, People's Republic of China, in 1990 and has since been maintained in unialgal culture in a cylinder system (Lignell et al., 1987) at 22°C and 33% salinity with 16 h of light, 150 μmol photons m^{-2} s^{-1}, and 8 h of darkness.

3.3. Enzyme activity assays

Activity of α-1,4-glucan lyase was measured by addition of the enzyme (0.37 units/500 μl assay) to maltose, amylopectin or soluble starch dissolved in 50 mM MOPS–NaOH, pH 6.2, equilibrated to and incubated at 30°C. The standard reaction volume was 100 μl. The reaction was stopped by heating the solution to 100°C for 2 min. One unit of α-1,4-glucan lyase activity was defined according to Yu et al. (1993) as the amount of enzyme that at 30°C liberates 1 μmol of 1,5-anhydrofructose min^{-1} from 6 mg soluble starch in 300 μl. The linearity of the reaction was assured by examination of the reaction at several time points. Maltose was degraded by α-1,4-glucan lyase into equimolar ratios of 1,5-anhydro-D-fructose and glucose and the latter was quantified as a measure of enzyme activity. When longer molecules were used as substrate, the 1,5-anhydrofructose removed from the non-reducing end of the chain was measured in order to determine enzyme activity.

3.4. Glucose detection

Glucose was quantified using the GOD-PAP kit (Merck). To 0.1 ml of the enzyme activity assay the GOD-PAP solution (1 ml) was added, mixed and incubated at 30°C for 30 min. The absorbance was measured at 490 nm with a 96-well plate reader and the amount of glucose was determined by comparison against a standard curve. None of the compounds used for α-1,4-glucan lyase inhibition had any effect on the GOD-PAP reagent enzymes, neither did the pH of the enzyme assay.

3.5. 1,5-Anhydro-D-fructose detection

For detection of 1,5-anhydro-D-fructose 300 μl of dinitrosalicylic acid solution (DNS) prepared according to Steup (1990) was added to 300 μl of the enzyme reaction and incubated at room temperature for 15 min. The absorbance at 546 nm was measured and compared to a standard curve made with purified 1,5-anhydro-D-fructose. The standard concentration of maltose was 4 mg/ml (11.1 mM), amylopectin 6 mg/ml and of soluble starch 20 mg/ml.

3.6. NMR spectroscopy

^{1}H NMR spectra were recorded with a Varian VXR-400 instrument at a probe temperature of 30°C at 400 MHz and the chemical shifts are given relative to internal TSP (sodium 3-trimethylsilyl-propionate, δ 0.00). Maltose (10 mg/ml = 28 mM) was dissolved in D_2O and allowed to equilibrate by mutarotation over night, yielding ca. 60% β- and 40% α-maltose. α-1,4-Glucan lyase (12.3 units) was added and the reaction was followed over 50 min. The chemical shifts of the signal of H-1 of the non-reducing residue for both α- and β-maltose was δ 5.44 and of H-1 of the reducing residue δ 5.26 and 4.68 for the α- and β-form, respectively.

The chemical shift of the signal of H-1 for β-glucose was δ 4.67 while that of α-glucose was coincident with that of α-maltose (δ 5.26). The amount of β-glucose and β-maltose could be estimated directly from the integrated peaks of the chemical shifts δ 4.67 and 4.68, respectively. The amount of α-maltose, however, had to be calculated by subtracting the value of the integrated peak at δ 4.68 (β-maltose) from the value of the integrated peak at δ 5.44 (β-maltose and α-maltose) (Eq. (1)) and α-glucose was then calculated by subtracting the calculated value of α-maltose from the peak at δ 5.26 (α-maltose and α-glucose) (Eq. (2)).

$$\alpha - \text{maltose} = \delta\ 5.44 - \delta\ 4.68 \quad (1)$$

$$\alpha - \text{glucose} = \delta\ 5.26 - (\delta\ 5.44 - \delta\ 4.68) \quad (2)$$

3.7. Determination of kinetic constants

K_m and V_{max} values for maltose were determined under initial velocity conditions. The direct linear plot (Eisenthal and Cornish-Bowden, 1974) was used to

determine the K_m and V_{max} for the different reactions and the values were compared to those determined by Lineweaver-Burke, Hanes and Hofstee graphs. The dissociation constants of the enzyme inhibitor complex, K_i and K_i' were determined from secondary plots of the steady-state parameters. A minimum of four different concentrations of inhibitor was used for the calculations of inhibitor constants.

3.8. Effect of pH

For the determination of K_m and V_{max} at different pH values maltose was dissolved in 100 mM of the appropriate buffer supplemented with 0.5 M NaCl. The buffers were glycine–HCl (pH 2.0–3.5), citric acid–NaOH (pH 4.0–5.0), MOPS–NaOH (pH 6.2–7.2) and Bicine–NaOH (pH 7.6–8.4). The addition of 0.5 M NaCl had no effect on the activity of the α-1,4-glucan lyase.

3.9. Modification of α-1,4-glucan lyase by EDAC and nucleophiles

All inactivation experiments were performed at 30°C with α-1,4-glucan lyase (0.09 U/10 μl inactivation assay) in 200 mM MES (pH 4.8) with 250 mM GEE (glycine ethyl ester) and 0–200 mM EDAC (1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide), unless specified otherwise. Both EDAC and GEE were dissolved in water immediately before use and inactivation was initiated by the addition of EDAC. A control experiment of enzyme and nucleophile (in our experiment GEE) in buffer was run simultaneously and corresponded to 100% activity. The effect of ligands on the inactivation was performed at concentrations corresponding to 10 apparent K_m or 10 apparent K_i. The chemical modification was stopped by a 100-fold dilution of the proteins in a solution of 100 mM MOPS–NaOH (pH 6.2) and 15 mM maltose in 100 μl. Enzyme activity was determined from the production of glucose. The inactivation kinetics were fitted to Eq. (3)

$$\log(\%\ \text{residual activity}) = -k_i \cdot t \quad (3)$$

where k_i is the pseudo-first order inactivation rate constant for a given concentration of EDAC and t the time of inactivation for which the residual activity is measured. The inactivation order (n) was calculated according to Eq. (4).

$$\log k_i = n \cdot \log[\text{inactivator}] + \log k_i' \quad (4)$$

where k_i' is the second order inactivation rate constant. The effect of pH on the modification was examined in MES–NaOH buffer (pH 4.5–6.0). The pK_a of the amino acid residue modified by EDAC was evaluated using Eq. (5).

$$1/k_i = K_a/[H^+]k_m + 1/k_m \quad (5)$$

where k_m is the maximal pseudo first-order inactivation rate constant and K_a is the ionisation constant of the modified residue.

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