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Enhancement of pH-stability of a low molecular mass endoglucanase from *Fusarium oxysporum* by protein pegylation

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Abstract

The stability of the low molecular mass endoglucanase (23.2 kDa) from *Fusarium oxysporum* at alkaline pH was enhanced by chemical modification. Two distinct types of amino acid-specific modifiers were used. The first, either cyanuric chloride activated polyethylene glycol (CC–PEG) or polyethylene glycol succinimidyl succinate active ester (SS–PEG), react (more or less specifically) with protein amino groups. The second type, maleimide polyethylene glycol (Mal–PEG), is specific for cysteinyl residues. The enzyme lost almost all of its activity when modified with CC–PEG, whereas no inactivation was observed with SS–PEG and Mal–PEG. The modified endoglucanase showed remarkably enhanced alkaline pH stability. When acting upon cello-oligosaccharides and 4-methylumbelliferyl cello-oligosaccharides, the enzyme preferentially cleaved the internal glycosidic bonds. The modified enzymes mediated a decrease in the viscosity of carboxymethyl cellulose (CMC) associated with the release of only small amounts of reducing sugar. Thus, the modified enzyme retains the endo character of the native enzyme.

Keywords: Endoglucanase; pH-Stability; Chemical modification; Polyethylene glycol

1. Introduction

Endo-(1→4)-β-D-glucanases (EC 3.2.1.4) cleave β-linked bonds of the cellulose molecule. The cellulolytic enzymes have recently been the focus of much attention because of their potential use in laundry detergents [1]. One of the most important objectives of enzyme engineering is to enhance the stability of enzymes. In particular, the pH stability of enzymes used in laundry detergents (lipases, proteases, amylases, cellulases) is an important factor in the overall process productivity. Protein stabilization has been achieved by several methods [2], including immobilization, site-directed mutagenesis, chemical modification, and cross-linking. Enzymes

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from alkalophilic microorganisms can evidently be selected.

Chemical modification of an enzyme usually leads to an alteration of its surface structure and can thus be used to impart desired properties to the biocatalyst [3]. Polyethylene glycol (PEG), a synthetic polymer with amphipathic properties, is water soluble, lacks toxicity, is highly flexible and its well-defined chemistry makes it ideally suited for many cross-linking or tethering applications. In contrast to the vast number of studies on synthetic or therapeutic applications of PEG-modified enzymes, the effect of such modification on enzyme stability has been addressed by only a few researchers [3,4]. The following PEG derivatives have been reported:

Sulfhydryl-selective PEG: methoxypolyethylene glycol–maleimide (Mal–PEG).—Reaction of maleimide with protein thiol groups is a particularly useful reaction for the preparation of bioconjugates. It is highly specific and occurs under mild conditions. Well-defined, bioactive PEG–protein conjugates have been prepared in this way [5,6].

Lysine-active PEGs.—*PEG–succinimidyl succinate*. The frequently used *N*-hydroxy-succinimidyl active ester of PEG succinate (SS–PEG) [7,8] reacts with protein amino groups under mild conditions, within short periods of time yielding extensively modified, yet active, conjugates. A potential problem with this derivative is that the ester linkage in the backbone is susceptible to hydrolytic cleavage.

PEG–cyanuric chloride. Enzyme–PEG conjugates are formed by reaction of PEG-linked cyanuric chloride (CC–PEG) with protein amino groups [9,10]. This method of polyethylene glycol modification of enzymes has been studied by several researchers [11–13]. However, cyanuric chloride has the disadvantage of being very toxic and generally gives rise to high molecular weight cross-linked reaction products [14,15].

In the present work, we have modified the surface of the low molecular endoglucanase of *Fusarium oxysporum* by covalent protein pegylation with the above PEG derivatives specific for lysine or cysteine attachment. The effect of these modifications on the activity, pH-stability and mode of action of the enzyme has been further analyzed.

2. Experimental

Materials and methods.—*Chemicals*. Sephacryl S-100 was purchased from Pharmacia (Sweden). Cello-oligosaccharides and methoxypolyethylene glycol activated with cyanuric chloride (CC–PEG) were purchased from Sigma (USA) while methoxypolyethylene glycol–maleimide (Mal–PEG) and *N*-hydroxy-succinimidyl ester of methoxypolyethylene glycol succinate (SS–PEG) were from Shearwaters Polymers (USA). The average molecular weight of all polyethylene derivatives was 5 kDa.

Production and purification of endoglucanase. The wild-type strain F3 of *F. oxysporum*, isolated from cumin [16], was used for the production of the enzyme. The stock culture was maintained at 4 °C on potato dextrose agar. For the production of the endoglucanase, *F. oxysporum* was cultured in a 20 L (17 L working volume) stirred tank fermentor for 4 days at 30 °C and pH 6.0 using a previously described salts medium [17] supplemented with 2% w/v of alkali-treated cellulose 123 (Schleicher & Schull, Dassel, Germany) as carbon source. The low molecular mass endoglucanase was purified to homogeneity by gel filtration and anion exchange chromatography as described previously [18].

Enzyme assay. Endo-β-(1→4)-glucanase activity was determined in 50 mM sodium phosphate buffer, pH 7, at 50 °C using 1.0% CMC as substrate. The release of reducing sugars (after 15 min incubation) was determined by the 3,5-dinitrosalicylic acid (DNS) method [19] with one unit (U) defined as the amount of enzyme liberating 1 μmol/min of reducing sugars.

The relationship between the change in fluidity and reducing sugars, released from a CMC solution by the endoglucanase, was determined as follows: a 25 mL reaction mixture containing 4% (w/v) CMC (low viscosity), and the enzyme (7 μg) in 0.01 M citrate buffer pH 6.0 was incubated at 37 °C for a period of 2 h. At different time intervals, the amount of

reducing sugars released was measured along with the viscosity of the reaction mixture (Oswald viscometer). The reciprocal of the specific viscosity ($1/n_{sp}$) was calculated from the formula $1/n_{sp} = t_o/(t - t_o)$, where t and t_o represent the time of outflow of the reaction mixture and buffer, respectively. This was plotted against the reducing sugars released.

Activity towards Glc_n and MeUmb$(Glc)_n$. Reaction mixtures (1.0 mL) consisting 0.9 mL of 20 mM Glc_n; $n = 2–5$, in 0.1 M sodium acetate buffer, pH 6.0, and 0.1 mL enzyme (10 μg protein) were incubated at 40 °C for 1 h. Activity towards MeUmb$(Glc)_n$; $n = 2–6$, (2 mM) was determined and analyzed under identical conditions as above, except that 1 μg of protein was used and the reaction was performed at 25 °C. Samples (50 μL) were removed at different time intervals and analyzed by HPLC using NH_2-μBondapak column (300 × 3.9 mm) (Waters) with acetonitrile:water (7:3) as mobile phase at a flow rate of 1.0 mL/min. Glc and oligosaccharides were detected by a Waters R 401 differential refractometer, while the MeUmb and MeUmb$(Glc)_n$; $n = 2–6$, were identified using a Waters UV detector (model 440) at 313 nm. The products were quantified on the basis of peak height using standard $(Glc)_n$, MeUmb and MeUmb$(Glc)_n$ solutions. Analysis of the frequency of bond cleavage of MeUmb$(Glc)_n$ was carried out as described previously [20].

Determination of pH-stability. The pH-stability was determined by incubating the enzyme in the pH region 7.0–12.0, at 30 °C for 24 h and measuring the residual activity at pH 7 (see above). The following buffering systems were used: 0.1 M phosphate (pH 6–8), 0.1 M Tris–HCl (pH 9.0), 0.1 M glycine–NaOH (pH 10), 0.1 M KCl–NaOH (pH 11–12).

Endoglucanase modification. A 1 mg sample of purified endoglucanase was dissolved in 1 mL of phosphate buffer at pH 8. Then 50 mg of CC–PEG, Mal–PEG or SS–PEG were added to the solution and the mixture was incubated under mild shaking at 30 °C for 1 h. The mixture was applied to a gel filtration column, Sephacryl S-200 column (2.5 × 100 cm), equilibrated with 10 mM phosphate buffer, pH 7.0 in order to remove the non-reacted PEG-derivative. The endoglucanase active fractions were pooled and concentrated using an Amicon PM-10 membrane system.

3. Results and discussion

Effect of chemical modification on endoglucanase molecular mass and activity.—Compared to the native enzyme, an average increase of 30–40 kDa was determined for the molecular weight of the modified endoglucanases in the Sephacryl S-200 column, corresponding to an attachment of 6 to 8 PEG chains per protein molecule (average molecular weight of 5 kDa for all PEG derivatives). These values however, should not be considered as an accurate estimate of the number of PEG chains attached to the protein molecule. Protein pegylation, usually leads to an increase of the hydrodynamic volume, resulting in increased observed resident times during gel filtration of the PEG-modified proteins. [15,21].

Almost total activity loss occurred when the enzyme reacted with CC–PEG, while there was practically no inactivation during reaction with SS–PEG and Mal–PEG (92 and 88% residual activity, respectively). Cyanuric chloride is very toxic and requires safety measures for its manipulation [14,15]. It is also important to note that the cyanuric chloride procedure gives heterogeneous reaction products which consist of a mixture of mono-, di- and tri-mPEG triazine ether derivatives [22]. Finally, the cyanuric chloride procedure of activation cannot be used with PEG because this leads to polymerization of the PEG. However, the use of succinimidyl succinate and maleimide as the reagents for the activation of PEG is safe and gives well-characterized products.

Stability of the modified low molecular endoglucanase against alkaline environment.—The modified endoglucanase was always more stable than the native enzyme under extreme pH conditions (Fig. 1). More specifically, at pH 12, 25 °C and 24 h incubation time, the native enzyme was completely inactivated while 66 and 73% of the initial activity remained in SS–PEG and Mal–PEG modified endoglucanase, respectively. These results

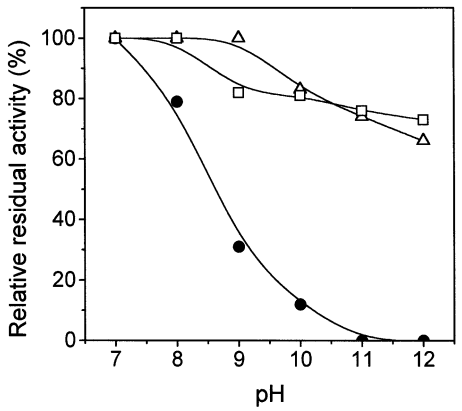

Fig. 1. pH-stability of the PEG-modified endoglucanases. Incubation time 24 h. ●, Native enzyme; □, Mal–PEG modified endoglucanase; △, SS–PEG modified endoglucanase.

showed that the modified endoglucanase was more stable against the reaction environment than the native cellulase because the copolymer modifier, which has long PEG chains, creates a buffering action against denaturation of enzyme [23].

Effect of chemical modification on the mode of action on CMC, Glc_n and MeUmb$(Glc)_n$.— The relationship between the change in specific fluidity and the release of reducing sugars during the hydrolysis of CMC by the modified endoglucanase is shown in Fig. 2. The rapid decrease in the viscosity of CMC (4% w/v) solution with the release of only small amounts of reducing sugar indicated that the modified enzyme catalyzed random cleavage of the above substrate similar to that of the native enzyme [18].

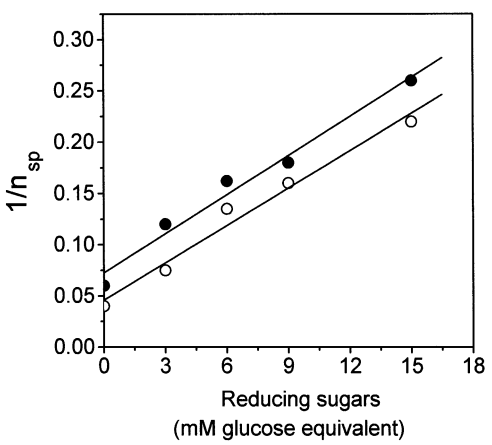

Fig. 2. Plot of increase in fluidity vs. the release of reducing sugars for the hydrolysis of CMC by the SS–PEG (○) and Mal–PEG (●) modified endoglucanase of *F. oxysporum*.

Table 1
Hydrolysis products released from cello-oligosaccharides by the SS–PEG and Mal–PEG modified endoglucanases from *F. oxysporum*

Substrate	Product molar ratio (%)			
	Glc_1	Glc_2	Glc_3	Glc_4
SS–PEG endoglucanase				
Glc_3	22	28	50	
Glc_4	29	48	22	1
Glc_5	16	52	32	0
Mal–PEG endoglucanase				
Glc_3	33	36	31	
Glc_4	16	52	32	0
Glc_5	12	50	38	0

The modified enzymes were active on Glc_n with at least three Glc residues. Analysis of product distribution revealed that the modified enzymes preferentially cleaved the internal glycosidic bonds of Glc_3, Glc_4 and Glc_5 (Table 1).

MeUmb$(Glc)_n$ were used to localize the position of the cleaved glucosidic linkage as well as to calculate the relative rates of cleavage for each linkage under unimolecular hydrolysis conditions (i.e., the bond cleavage frequency). The modified enzymes were inactive on MeUmbGlc but active on higher cello-oligosaccharides of MeUmb (Figs. 3 and 4). They preferentially cleaved the first glucosidic bond of MeUmb$(Glc)_2$ and MeUmb$(Glc)_3$ from the aglycon. However, as the chain length of the substrate was increased, the enzyme showed a strong preference for the internal glycosidic linkages. The bond cleavage frequency distribution also revealed that the

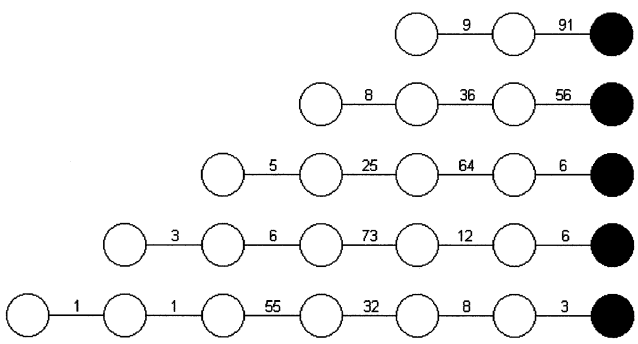

Fig. 3. Bond cleavage frequency for the hydrolysis of MeUmb-$(Glc)_n$ by the Mal–PEG endoglucanase of *F. oxysporum*. ○, Glc; ●, MeUmb.

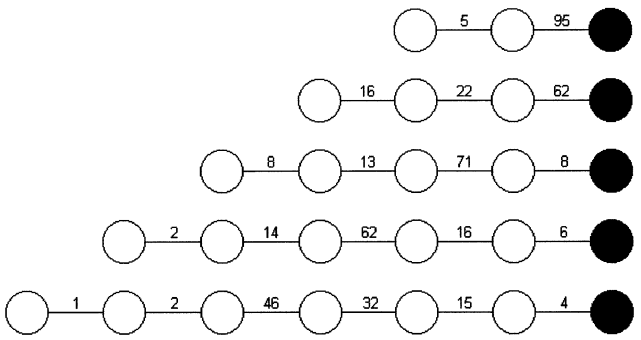

Fig. 4. Bond cleavage frequency for the hydrolysis of MeUmb-$(Glc)_n$ by the SS–PEG endoglucanase of *F. oxysporum*. ○, Glc; ●, MeUmb.

preferential site of attack shifts to the internal linkages with increasing size of the oligomer, a feature shared for many endoglucanases.

Conclusions

The low molecular mass endoglucanase from *F. oxysporum* was chemically modified by two distinct types of amino acid-specific modifiers, cyanuric chloride activated polyethylene glycol (CC–PEG) and polyethylene glycol succinimidyl succinate active ester (SS–PEG) specific for lysine attachment and maleimide polyethylene glycol (Mal–PEG) specific for cysteine attachment. Almost total activity loss occurred in the case of enzyme reaction with CC–PEG. In contrast there was no inactivation after enzyme reaction with SS–PEG and Mal–PEG. The modified endoglucanase showed remarkably enhanced stability against alkaline pH. Analysis of reaction mixtures by high pressure liquid chromatography revealed that the modified enzyme cleaved preferentially the internal glycosidic bonds of cellotetraose, cellopentaose and CMC. Thus the modified enzyme retains the endo character of the native enzyme.

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