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Partial Purification and Properties of Phleinase Induced in Stem Base of Orchardgrass after Defoliation

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ABSTRACT

Phleinase induced in stem base of orchardgrass (*Dactylis glomerata* L.) after defoliation was partially purified with ammonium sulfate precipitation, DEAE-Sephadex chromatography, gel filtration, and preparative polyacrylamide gel electrophoresis. The molecular weight of phleinase was 57,000 as determined by gel chromatography. The enzyme showed normal Michaelis-Menten kinetics and its K_m value was 91 millimolar for phlein of mean degree of polymerization 60 as substrate. Reaction velocity of the enzyme was proportional to molarity of phlein irrespective of its chain length (mean degree of polymerization, 30 to 314). Phleinase attacked terminal fructosyl linkage of phlein by multi-chain mechanism. Phleinase cleaved β-2,6 linkage, β-2,6 linkage branched with β-2,1 linkage, and β-2,1 linkage of fructan in order of affinity, but not sucrose. Phleinase exhibited an optimum activity at pH 5.5 at 40°C. Its complete inactivation occurred at 60 and 70°C without and with phlein, respectively. Heat inactivation of the enzyme was enhanced by *p*-chloromercuribenzoate and protected partially by L-cysteine. The enzyme was inhibited by sulfhydryl reagents such as *p*-chloromercuribenzoate and Hg^{2+}. The modes of action of phleinase were compared with those of the related enzymes.

Grasses of temperate origin accumulate fructan as a principal available carbohydrate in the stem bases. The fructan in the grasses consists of linear chain of β-2,6 linkage with a D-glucose molecule at its end, and is designated as phlein or grass levan for discriminating from inulin, β-2,1-fructan, and bacterial levan (1, 6). The degree of polymerization of the phlein molecule varies with seasons, growth conditions, and grass species (15).

The phlein reserved in the stem bases is degraded intensively in response to defoliation and used for the vigorous regeneration of new shoots. Several workers have suggested that the rate of regrowth following defoliation is related to the level of available carbohydrates in the stem base (2, 8, 21). In the previous papers, we have reported that phleinase is induced in the stem bases of timothy and orchardgrass after defoliation, and the change in phlein concentration is inversely related to the fluctuation in phleinase activity (13, 23). There have been some reports on the properties of levanases produced by *Arthrobacter tumescens* (24) and *Pseudomonas* sp. (5), and inulinases by Jerusalem artichoke (10) and *Saccharomyces fragilis* (16). However, little is known about the nature of phleinase in grasses. This study is concerned with the properties of phleinase induced in the stem base of orchardgrass after defoliation.

MATERIALS AND METHODS

Plant Materials. A 4-year-old stand of orchardgrass (*Dactylis glomerata* L. cv Aonami) established on the experimental field of National Grassland Research Institute was used. The first, second and third cuttings were conducted at about 5 cm above ground level on May 7, June 10, and July 22, respectively. Ammonium sulfate (250 kg/ha) was applied after the second cutting. On July 25, the stubbles were dug out and the vegetative stems of 2.5 cm from the base were collected.

Preparation of Crude Enzyme. Two hundred and fifty g of the stem bases were cut into slices (about 2 mm in thickness) and homogenized in 4 volumes of cold 0.1 M K-phosphate (pH 7.2) containing 5 mM β-mercaptoethanol with 10% insoluble PVP for 2 min using a Waring Blendor. The homogenate was squeezed through four layers of gauze and centrifuged at 14,000 *g* at 4°C for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant and the precipitate between 40 and 70% saturation was collected by centrifugation. The precipitate was dissolved in 20 ml of 20 mM K-phosphate (pH 7.2) and filtrated on a column of Sephadex G-25 (2 × 26 cm) with the same buffer as eluant. All subsequent procedures were carried out at about 4°C. The protein fractions were combined and dialyzed against two changes of 20 volumes of 50 mM Na-acetate buffer (pH 5.4) for 4 h with stirring. The protein precipitated during dialysis was removed by centrifugation and the supernatant was used as the starting material for further purification.

Preparation of Phlein and Triticin. Two hundred g of the stem bases of orchardgrass collected in late November were cut into slices and homogenized in 3 volumes of cold 10 mM β-mercaptoethanol solution. The homogenate was squeezed through gauze, and the filtrate was centrifuged at 14,000 *g* at 4°C for 15 min. Each of 50 ml of 5% $ZnSO_4 \cdot 7H_2O$ and 0.3 N $Ba(OH)_2$ was successively added to the supernatant, and the resulting precipitate was removed by centrifugation. Four volumes of ethanol were added to the supernatant to collect the precipitate by centrifugation. The precipitate was dissolved in 200 ml of distilled H_2O in a boiling water bath for 3 min and cooled in running tap water, followed by centrifugation. The supernatant was treated with 20 g of the mixture of Amberlite IR-120(H^+) and IRA-45(OH^-), and shaken occasionally for 30 min at room temperature, followed by filtration through a filter paper *in vacuo*. Four volumes of ethanol were added to the filtrate and the resulting precipitate was collected by centrifugation. The precipitate was suspended in 150 ml of 80% ethanol (v/v) for centrifugation. This procedure was repeated four times. The precipitate was preliminarily dried at room temperature for further lyophilization. Phlein thus prepared showed little reducing power. Fructose and a trace of glucose were detected on a paper chromatogram of the hydrolysate of the phlein. No reducing sugars were produced by treatment of α-amylase. Triticin was prepared from rhizomes of quackgrass (*Agropyron repens* [L.] P. Beauv.) by the method of Arni and Percival (4).

Fractionation of Phlein. Two g of phlein dissolved in 15 ml of distilled H_2O were chromatographed on a column of Sephadex

G-100 (2.6 × 70 cm) at a flow rate of 60 ml/h with distilled H_2O as eluant, and the eluate was collected in 10-ml fractions. This procedure was repeated and the fractions of the same elution volume were combined. Each fraction lyophilized was dissolved in 5 ml of distilled H_2O and chromatographed again on the same column. The IR spectrum of each phlein fraction was identical with that reported by Suzuki (18). $\overline{DP}$[1] of each fraction was estimated by the ratio of reducing sugars to glucose in the hydrolysate with 0.7 N HCl in a boiling water bath for 10 min. When the logarithm of the mol wt calculated from $\overline{DP}$ of each phlein fraction was plotted against the elution volume, a nearly straight line was obtained.

Assay of Enzyme Activities. Phleinase activity was assayed by measuring the production of reducing sugars from phlein. The reaction mixture consisted of 4 mg of phlein ($\overline{DP}$ 60) in 150 μl of McIlvaine buffer (pH 5.5) and 50 μl of enzyme solution, unless otherwise stated. The reaction mixture was incubated at 30°C for 30 min or 1 h, followed by heating in a boiling water bath for 1 min. The amount of reducing sugars produced was determined by the method of Somogyi (17) and evaluated as fructose equivalent. One unit of phleinase activity was defined as the amount of enzyme liberating 1 μmol fructose/h under the reaction conditions described.

The reaction mixture for invertase and α-glucosidase consisted of 1 ml of 0.1 M sucrose and methyl-α-D-glucoside in McIlvaine buffer (pH 5.5), respectively, and 0.2 ml of enzyme solution. After incubation at 30°C for 1 h, the mixture was neutralized with 1 N NaOH, and the amount of reducing sugars produced was determined.

α-Glucosidase activity was also assayed using *p*-nitrophenyl-α-D-glucoside. The reaction mixture (0.8 ml of 25 mM substrate in McIlvaine buffer [pH 5.5] and 0.2 ml of enzyme solution) was incubated at 30°C for 30 min, followed by addition of 2 ml of 0.2 M sodium carbonate. The amount of *p*-nitrophenol liberated was measured by the *A* at 400 nm.

Disc Electrophoresis. Disc electrophoresis was conducted by the method of Davis (9) with 7.5% polyacrylamide gel and 5 mM Tris-38 mM glycine buffer (pH 8.3). Samples containing 50 μg protein in 0.5 M sucrose were layered onto the gel for electrophoresis at 2 mamp/tube under cold condition. The gel was stained with 1% amide black, and scanned at 640 nm with a Yamato densitometer, SD-92, after destaining. The gel was also cut into slices (2 mm in thickness) which were immersed separately in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.2). After standing overnight at 4°C, the enzyme activity in the buffer solution was assayed.

Double Immunodiffusion Test. Rabbits were immunized with the finally purified fraction (100 μg protein in complete Freund's adjuvant/rabbit) by intramuscular injections at intervals of 3 weeks. The antiserum was obtained 10 d after the third injection and the globulins precipitated by 30% $(NH_4)_2SO_4$ saturation were dialyzed against 0.15 M borate-NaOH buffer (pH 8.0). The specificity of the globulin fraction was tested by Ouchterlony double immunodiffusion technique. Cross-reaction among the globulin fraction, crude extract, and the antigen were tested.

Mol Wt Determination. The mol wt of phleinase was determined by the method of Andrews (3) with a Sephadex G-100 column (1.5 × 60 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl using ribonuclease A, chymotrypsinogen A, egg albumin, and BSA as the authentic specimens.

Gel Chromatography of Degradation Products of Phlein. Degradation products of phlein were loaded onto a Sephadex G-75 column (1.5 × 60 cm) equilibrated with distilled H_2O and eluted with water at a flow rate of 21 ml/h. The eluate was collected in 3-ml fractions. The amount of total sugars in the eluate was determined by anthrone reagent (19).

Paper Chromatography of Sugar. Fractions 37 to 42 of the gel chromatography of phlein hydrolysate were combined and lyophilized. The lyophilizate was dissolved in 2 ml of distilled H_2O and chromatographed on a filter paper of Whatman 3 MM by ascending method using *n*-propanol:ethyl acetate:H_2O (7:1:2, v/v) as solvent. The sugar was detected with silver nitrate (20) and orcinol (7) reagents.

Glucose and Protein Determinations. Glucose was determined using a glucose oxidase-peroxidase reagent (22). Protein was measured by the method of Lowry *et al.* (11) or *A* at 280 nm using BSA as standard.

Chemicals. Glucose oxidase-peroxidase reagent and invertase were obtained from Boehringer Mannheim; proteins used as authentic specimens and standard, *p*-nitrophenyl-α-D-glucoside, and insoluble PVP were from Sigma; inulin was from Merck. Sephadex G-25, 75, and 100, and DEAE-Sephadex A-50 were the products of Pharmacia.

RESULTS

Purification of Phleinase. Since a preliminary experiment showed that phleinase was not adsorbed to DEAE-Sephadex at pH 4.5, the eluant system around pH 5 was employed for the purification of phleinase by anion chromatography. The crude enzyme preparation was applied to a DEAE-Sephadex A-50 column (2 × 21 cm) equilibrated with 50 mM Na-acetate buffer (pH 5.4). After washing the column with the acetate buffer, phleinase was eluted in the same buffer with a linear gradient of NaCl from 0 to 0.25 M at a flow rate of 18 ml/h, and the eluate was collected in 8-ml fractions. As shown in Figure 1, phleinase activity was eluted as one peak. Fractions 50 to 56 were combined and concentrated to about 5 ml *in vacuo* using a collodion bag. The concentrate was dialyzed against 1 L of 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl.

The dialysate was loaded onto a Sephadex G-100 column (1.5 × 60 cm) equilibrated with the Tris-HCl buffer containing 0.1 M NaCl and eluted with the same buffer at a flow rate of 9 ml/h. One peak of phleinase activity was obtained. The active fractions were combined and concentrated to about 7 ml as above. The concentrated enzyme solution was dialyzed against 52 mM Tris-glycine buffer (pH 8.9).

Preparative electrophoresis on polyacrylamide gel was conducted for further purification. The spacer and separation (7.5% acrylamide, pH 8.9) gels were prepared in the apparatus of Toyo company, CD-50, as described by Davis (9). The separation gel was in the shape of cylinder of 4.7 cm in diameter with a hollow

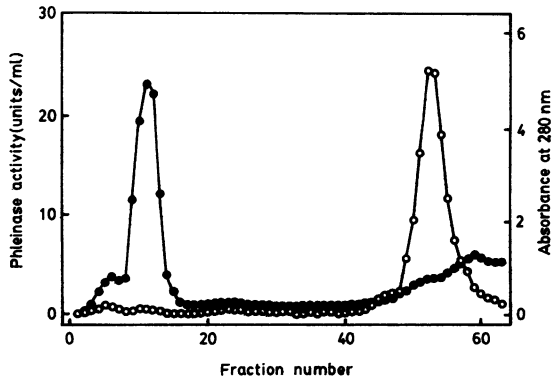

FIG. 1. Elution profile of 40 to 70% $(NH_4)_2SO_4$ fraction on a column of DEAE-Sephadex A-50. The bound proteins were eluted with a linear gradient of 0 to 0.25 M NaCl (fractions 30 to 63). (○), phleinase activity; (●), *A* at 280 nm.

[1] Abbreviations: $\overline{DP}$, mean degree of polymerization; *p*CMB, *p*-chloromercuribenzoate; SH, sulfhydryl.

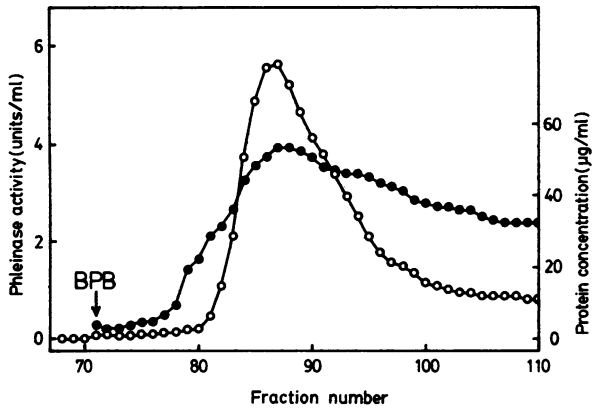

FIG. 2. Elution profile of Sephadex G-100 fraction by preparative PAGE. (○), phleinase activity; (●), protein concentration. BPB represents the position of bromephenol blue dye front peak.

of 2 cm in diameter and 3 cm in height. The upper and eluting buffers were composed of 52 mM Tris-glycine (pH 8.9) and 10 mM Tris-HCl (pH 8.1), respectively. The enzyme solution containing 0.1 M sucrose was layered onto the spacer gel and electrophoresis was performed at a constant current of 50 mamp under cold condition. The separated protein fractions were swept with the eluting buffer at a flow rate of 100 ml/h and the eluate was collected in 5 ml fractions. Elution diagram showed one peak of phleinase activity (Fig. 2). The fractions 83 to 95 were combined and concentrated to 4.7 ml as before. This enzyme solution was used for experiments of purity and properties of phleinase. A summary of the purification was presented in Table I.

Purity of the Final Preparation. In disc gel electrophoresis, the final preparation was stained as a relatively broad band holding a sharp dense streak in it, and the phleinase activity was detected coincident with the stained pattern (Fig. 3). Invertase activity was not detected in the final preparation. However, a double immunodiffusion test of the antiserum (obtained using the final preparation) against both the antigen and crude extract gave a spur-like precipitin line, indicating that extraneous antigenic principle(s) was presumably present in the final preparation.

Mol Wt and K_m Value of Phleinase. All of the phleinase activity was eluted in one peak by gel chromatography. The mol wt of phleinase was estimated to be about 57,000. The enzyme showed normal Michaelis-Menten kinetics. The K_m value for phlein ($\overline{DP}$ 60) was determined at pH 5.5 to be 91 mM by a Lineweaver-Burk plot.

Action Pattern of Phleinase. The mode of action of phleinase on phlein was examined in terms of the degradation products of phlein ($\overline{DP}$ 314) by the enzyme using gel and paper chromatography. Even after considerable degradation, fragments of phlein and saccharide cleaved from phlein were separately eluted as two discrete peaks, suggesting that phleinase hydrolyzes phlein by an endwise action (Fig. 4). When the fraction of low mol wt was chromatographed on a filter paper, only fructose was detected. From these results, phleinase is considered to cleave a terminal fructosyl residue from phlein.

Effect of Substrate Chain Length on Reaction Velocity. Reaction velocity was examined in connection with phlein chain length. When phlein differing in chain length was used at an equal w/v concentration, the velocity was in proportion to the molarity of phlein irrespective of its chain length ($\overline{DP}$ 30 to 314) (Fig. 5). When the substrate was used at equimolarity, all the reaction velocities were the same, suggesting that the reaction velocity of phleinase is considered to be proportional to the molarity of phlein irrespective of its chain length.

Effect of Substrate Chain Length on Sucrose Release. Release of sucrose and reducing sugar during degradation of phlein by phleinase were examined using the substrate differing in chain length. The amount of sucrose released was estimated from that of glucose using invertase. Sucrose was released at the late stage of degradation of short chain phlein, while no sucrose was detected during the reaction time in case of long chain phlein (Fig. 6). On the other hand, α-glucosidase was not detected in the enzyme solution, suggesting that phleinase attacks phlein by multi-chain mechanism.

Substrate Specificity. Phleinase affinity to fructan of different chain linkages was examined. As shown in Table II, phleinase produced more reducing sugar from phlein and triticin than from inulin. The enzyme hydrolyzed phlein more than triticin, β-2,6-fructan branched with β-2,1 linkage. These results suggest that phleinase cleaves fructans of β-2,6 linkage, β-2,6 linkage branched with β-2,1 linkage and β-2,1 linage in order of affinity. On the other hand, cleavage of β-2:α-1 linkage in sucrose by the enzyme was not detected.

Effects of pH and Temperature on Phleinase Activity. Phleinase exhibited an optimum activity at pH 5.5 and 40°C in citrate-dibasic sodium phosphate buffer using phlein as substrate. The enzyme activity with substrate phlein decreased drastically at 55°C and was completely lost at 70°C for 15 min at pH 5.5. On the other hand, in the case of the enzyme solution alone, slight and complete inactivations were observed at 40 and 60°C, respectively. These differences in response to temperature imply thermostabilization of the enzyme by the substrate.

Effects of Metal Ions, SH Reagents, and a Chelator. Among the metal ions examined, only Hg^{2+} completely inhibited phleinase activity. Metal ions except Hg^{2+} scarcely inhibited the activity. *p*CMB and EDTA inhibited the activity potently (92% at 0.1 mM) and slightly (20% at 1 mM), respectively.

Effects of *p*CMB and Cysteine on Thermostability. The inactivation of phleinase by heating was accelerated by 2 μM *p*CMB, while that was partially protected by 0.5 M L-cysteine. These results suggest that the SH group is involved in phleinase activity.

DISCUSSION

A comparison between the mode of action of the phleinase in orchardgrass and the related enzymes reported earlier will be discussed first here. The levanases from *Pseudomonas* sp. (5) and *Arthrobacter tumescens* (24) have been reported to liberate levanbiose and oligosaccharides, differing from phleinase in our study. Phleinase released the terminal fructose residue from phlein. Accordingly, the mode of action of phleinase is rather analogous to that of inulinase from Jerusalem artichoke (10) and

Table I. *Purification of Phleinase from Stem Base of Defoliated Orchardgrass*

Fraction	Vol	Protein	Activity	Specific Activity	Purification	Recovery
	ml	*mg*	*units*	*units/mg protein*	*-fold*	*%*
Crude extract	930	3450	5340	1.5	1	100
$(NH_4)_2SO_4$	95	1480	5170	3.5	2.3	97
DEAE-Sephadex A-50	56	75.2	876	11.6	7.5	16
Sephadex G-100	18	18.1	716	39.6	26	13
Preparative electrophoresis	65	2.9	243	83.8	54	5

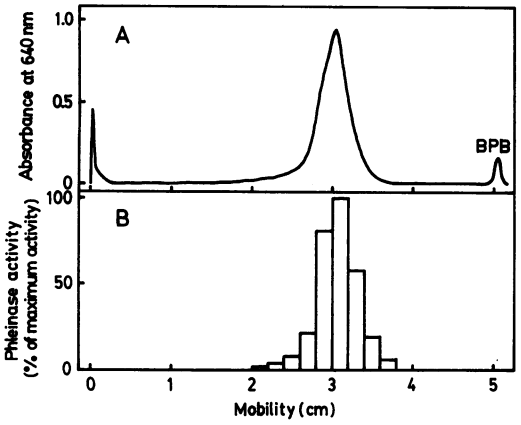

FIG. 3. Disc electrophoretic patterns of the fraction separated by preparative PAGE. A, Densitogram of protein stained with amide black at 640 nm. BPB indicates bromephenol blue dye front. B, Distribution of phleinase activity.

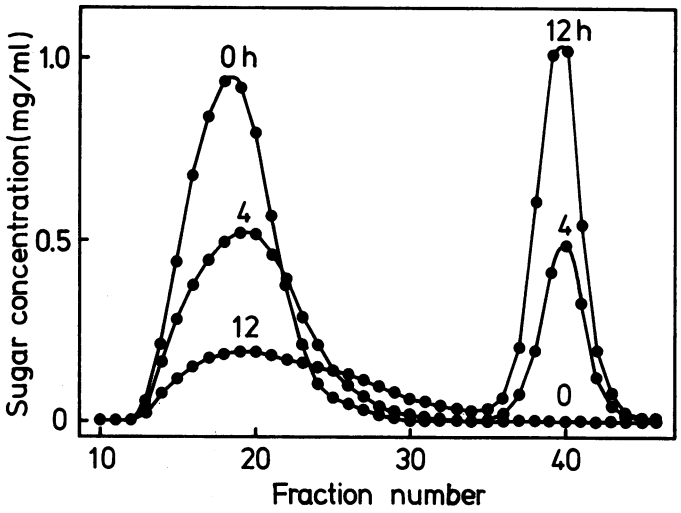

FIG. 4. Elution profiles of hydrolysates of phlein by phleinase on a column of Sephadex G-75. Reaction mixture consisted of 20 mg of phlein of $\overline{DP}$ 314 in 0.1 ml of McIlvaine buffer (pH 5.5) and 0.1 ml of enzyme solution, and was incubated at 30°C for 0, 4, and 12 h.

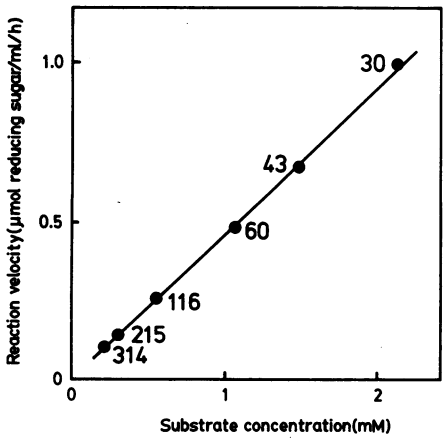

FIG. 5. Relationship between $\overline{DP}$ of phlein and reaction velocity of phleinase. Assayed at an equal w/v concentration of phlein. Reaction mixture consisted of 10.4 mg of phlein in 0.8 ml of McIlvaine buffer (pH 5.5) and 0.2 ml of 1:10 diluted enzyme solution. The number indicates $\overline{DP}$ of phlein.

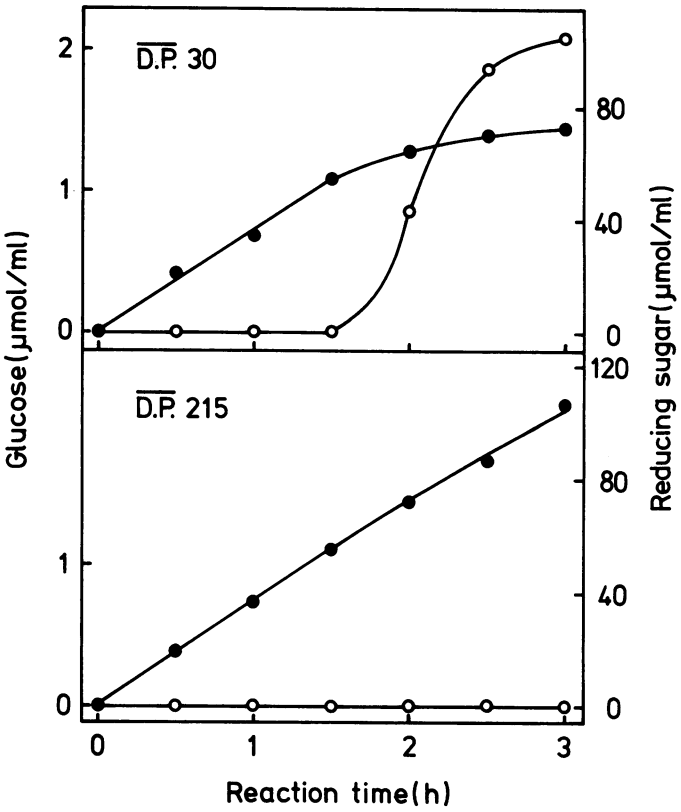

FIG. 6. Release of sucrose from short and long chain phleins by phleinase. Reaction mixture (0.86 μmol of phlein [$\overline{DP}$ 30 or 215] in 0.15 ml of McIlvaine buffer (pH 5.5) and 0.15 ml of enzyme solution) was incubated at 30°C. After termination of the reaction, 0.1 ml of invertase solution (1.5 U) was added to 0.1 ml of the above mixture and incubated at 30°C for 30 min for the determination of glucose amount.

Table II. *Substrate Specificity of Phleinase*

Substrate	Structure	Relative Activity[a]
		%
Phlein	Linear β-2,6-fructan	100
Inulin	Linear β-2,1-fructan	9
Triticin	Branched β-2,6-fructan with β-2,1 linkage	14
Sucrose	Fructosyl-glucoside(β-2:α-1)	0

[a] Assayed at an equimolar concentration (1 mM).

Saccharomyces fragilis (16), though the linkage of phlein (β-2,6) is different from that of inulin (β-2,1).

Inulinase from *S. fragilis* (16) was reported to hydrolyze inulin by single-chain mechanism, *i.e.* complete degradation of a substrate molecule before attacking another one. On the other hand, amyloglucosidase from *Aspergillus niger* was reported to hydrolyze malto-oligosaccharides by multi-chain mechanism, *i.e.* cleaving of the ends of the polymer chains in a completely random manner (14). In this respect, phleinase is assumed to attack phlein by multi-chain mechanism. The reason for this is that the terminal sucrose residue is released from short chain phlein only at the late stage of its degradation. Fructan such as phlein and inulin will be therefore of great advantage to the study of action mechanism for substrate degradation in that the substrate has a marker glucose residue at its end.

The present investigation showed that the velocity of fructose release from phlein by phleinase is proportional to the molarity of phlein irrespective of the chain length ($\overline{DP}$ 30 to 314), implying

that the reaction velocity depends on the number of terminal fructosyl β-2,6 linkages. Accordingly, the regrowth ability after defoliation is inferred to be closely related to the level of available carbohydrates in the stubble as was suggested by some workers (2, 8, 21).

The K_m value of phleinase for phlein was relatively large, indicating that the affinity of phleinase to phlein is low. This was also confirmed by our experiments (unpublished data) that purification of phleinase was carried out unsuccessfully by affinity chromatography using phlein as ligand. However, phlein concentration in the stem bases of orchardgrass and timothy after defoliation is inversely related to phleinase activity, indicating that phleinase is involved in the *in vivo* metabolism of phlein (13, 23). Fructose releasing velocity from phlein by phleinase was in proportion to the substrate concentration up to high molarity as revealed in K_m value. The concentration of phlein in the stem base of orchardgrass varies below 15% of fresh weight under usual growth condition. Supposing that $\overline{DP}$ of the phlein is 60, the molarity of phlein in the stem base is estimated below 15 mM, which is lower than the K_m value. This may reflect that the amount of fructose supplied for regrowth increases with increasing the molarity of phlein, in case phleinase activity is at the same level.

The present study dealt with phleinase from cytoplasm of orchardgrass. However, the previous paper indicated that high activity of phleinase is associated with the cell wall in timothy haplocorm (12). Further investigation will be necessary to clarify the properties of cell wall bound phleinase and the possibility of its release into cytoplasm.

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