[韓作誌(Korean J. Crop Sci.) 41(4) : 456~464(1996)]

Purification and Properties of Wheat Fructan Exohydrolase

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밀 Fructan Exohydrolase의 분리 및 특성

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ABSTRACT : Fructans are the major vegetative storage carbohydrate in wheat(*Triticum aestivum* L.). The depolymerization of fructans occurs by the sequential removal of terminal fructosyl residues by a specific fructan exohydrolase(FEH). The objective of this study was to isolate and characterize this enzyme in wheat. From stems and sheaths of field-grown wheat(cv. Clark), FEH was purified 356-fold using salt precipitation and a series of chromatographic procedures including size exclusion, anion exchange, and affinity chromatography. FEH had a molecular weight of 63.7 kD and an optima at pH 5.5 and 30℃. The K_ms for $\beta(2\rightarrow1)$ linked oligofructans varied, from 10 to 37mM, with the lowest K_m for tetrasaccharide. The V_{max} increased as degree of polymerization(DP) increased. Wheat FEH hydrolyzed only $\beta(2\rightarrow1)$ linked fructans but not $\beta(2\rightarrow6)$ linked timothy fructan or sucrose. The role of this FEH in fructan metabolism in wheat is discussed.

Key words : Wheat, Fructan exohydrolase, *Triticum aestivum*.

Fructans are the major storage carbohydrate of vegetative tissue in cereals and grasses of temperate origin.[14] The magnitude of fructan accumulation varies depending on the developmental stage and environmental conditions. Timothy(*Phleum pratense*) stem bases at anthesis may contain as much as 40% of their dry weight in fructans.[13] Similarly wheat stem internodes 24 days after anthesis have concentrations of soluble carbohydrates, largely fructan, in excess of 40% of the dry weight.[2] Fructans may accumulate to more than 70% of the dry weight in excised, illuminated primary leaf blades of barley(*Hordeum vulgare*).[21]

Frutan as the major storage reserve of vegetative tissues contributes carbohydrate to the ear during grain filling, particularly if photosynthesis were limiting.[12] The contribution of stored carbohydrates to grain yield has been assessed at 10 to 30% of the grain yield, although higher contributions(70%) have been suggested under extreme conditions in barley.[4,7] While fructans represent

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the carbohydrate reserve remobilized but are not transported themselves, their remobilization during the grain filling period can only occur after hydrolysis.

Fructan hydrolysis in plants can occur through the action of FEH(EC 3.2.1.80) or invertase(EC 3.2.1.26), both of which cleave the fructosyl linkages of fructans. Two forms of FEH, distinguished by their mobility on DEAE-cellulose columns, have been isolated from the tuber of Jerusalem artichoke(*Helianthus tuberosus*) and taproots of dandelion (*Taraxacum officinale*).[6,15] At least two FEH activities were also reported in crude extracts of leaves of *Lolium temulentum* and *Lolium rigidium*.[3,18] In the culm base of tall fescue(*Festuca arundinacea*), fructan hydrolase degrades phlein starting at the fructose end by exo-action.[19] The tall fescue enzyme is specific for the cleavage of $\beta(2\rightarrow6)$ linkages, but does not hydrolyze inulin. Yamamoto and Mino[23] also partially purified phleinase from the stem base of orchardgrass(*Dactylis glomerata*) following defoliation. The phleinase had higher affinity for $\beta(2\rightarrow6)$ linked fructan than $\beta(2\rightarrow1)$ linked fructan. Polyclonal antibody was raised against this purified phleinase and has shown that the increase in phleinase activity in stem base after defoliation was derived from *de novo* synthesis of the enzyme.[24] A partially purified FEH from barley, which like wheat contains branched fructan, was shown to be active against inulin fructans, but inactive against bacterial levan containing $\beta(2\rightarrow6)$ backbone and $\beta(2\rightarrow1)$ branches.[8]

Certainly to explain the value of fructan metabolism we must know more about the enzymology of fructan metabolism. Although FEH has been isolated and characterized from many plants, the enzyme has not been studied extensively in the cereals. The objective of this study was to isolate and characterize FEH in wheat.

MATERIALS AND METHODS

1. Enzyme purification

Wheat plants were grown at the Purdue Agronomy Research Center. Approximately one week prior to heading, leaf blades were excised to induce fructan hydrolysis. After one day, stems and sheaths were collected, frozen, and stored at −80℃ until used. One kilogram of tissue was cut into pieces(about 4cm) and ground in a blender in 1*l* of 50mM sodium acetate buffer, pH 5.0(buffer A). The resulting slurry was filtered through three layers of cheesecloth. The homogenate was centrifuged at 12,000g at 4℃ for 10min. Supernatants were fractionated with solid ammonium sulfate. Proteins precipitated by ammonium sulfate(30~70%) were resuspended in buffer A. Dissolved proteins were chromatographed on a Sephacryl S-200 column (2.5 × 50cm) and eluted with buffer A at a flow rate of 15.6ml/hr, with fractions collected every 10min. The fractions containing fructan hydrolytic activity were combined and applied to a DEAE/TSK column(1 × 25cm) pre-equilibrated with buffer A. After washing with three column volumes of buffer A, the DEAE/TSK column was eluted with a linear gradient of NaCl(0~0.1M in 100ml buffer A). Fractions containing fructan hydrolytic activity were pooled and applied to a Concanavalin A-Sepharose(ConA-Sepharose) column(1 × 9cm) equilibrated with 50mM sodium acetate(pH 5) containing 0.5M NaCl, 1mM $MnCl_2$, and 1mM $CaCl_2$(buffer B) and eluted with a linear gradient of α-methyl-

D-mannoside(0~50 mM in 100ml buffer B) after washing with two column volumes of buffer B.

2. Assay of enzyme activity

Five volumes of each fraction were mixed with one volume of 0.6M sucrose or Jerusalem artichoke fructan(DP>6, 40mg/ml fructose equivalents) to assay invertase and fructan hydrolytic activity, respectively. The enzyme mixtures were incubated at 30℃, and the reaction was stopped by mixing with 1ml of 0.1M imidazole buffer(pH 7.7) containing 0.45mM NADP, 0.9mM ATP, 5mM $MgCl_2$ and 0.2mg/ml BSA. The enzyme activities were calculated by quantitating glucose and fructose present in the reaction mixtures using a coupled enzyme assay.[11]

3. Preparation of substrate

To extract fructan from wheat, stem and sheath tissues were ground in liquid nitrogen with a mortar and a pestle. The tissue powder was extracted twice with boiling 76% (v/v) ethanol and then boiling water. The extracts were combined and flash evaporated. After concentrating a yellowish color developed which could be removed by passing the solution through a double bed ion exchange column of Dowex 1 and Dowex 50 and then through a C_{18} SepPac cartridge (Waters). Fructan from Jerusalem artichoke and timothy were extracted as above using tubers and haplocorms, respectively. Carbohydrates from each source were applied on a Bio-Gel P2 column(200~400 mesh, 2.5 × 45cm) and eluted with water at a flow rate of 10ml/h. The sugar in each 2ml fraction was measured with anthrone reagent.[10] Carbohydrates eluted early, known to contain more than 6 residues, were pooled and termed long-chain fructan. Inulins from dahlia and chicory were purchased from Sigma Chemical Company.

4. Molecular weight determination

The molecular weight of wheat FEH was determined by determining the retention time of proteins with known molecular weight on a Sephacryl S-200 column(2.5 × 45cm). β-Amylase(200kD), alcohol dehydrogenase(150kD), BSA(66kD), carbonic anhydrase(29kD), and cytochrome C(12.4kD) as standard markers were purchased from Sigma Chemical Company.

5. Determination of pH and temperature optima

To determine optimum pH of wheat FEH, pH was adjusted with MxIlvaine's buffer. Enzyme assays were conducted as described above. Since wheat FEH showed maximum activity at pH 5.5(Fig. 5), the enzyme activity was assayed at various temperatures at pH 5.5 to determine optimum temperature.

RESULTS AND DISCUSSION

Fig. 1 shows a Sephacryl S-200 chromatographic profile of FEH activity following fractionation with ammonium sulfate. Two invertase peaks differing slightly in molecular weight were eluted. Fructan exohydrolase coeluted with the second invertase peak both having a native molecular weight of about 64kD. The fractions containing both the second invertase peak and the FEH peak were pooled and applied to a DEAE/TSK column pre-equilibrated with 50mM Na-acetate buffer(pH 5.0) (Fig. 2). Fractions having FEH and invertase activity were eluted at a differ-

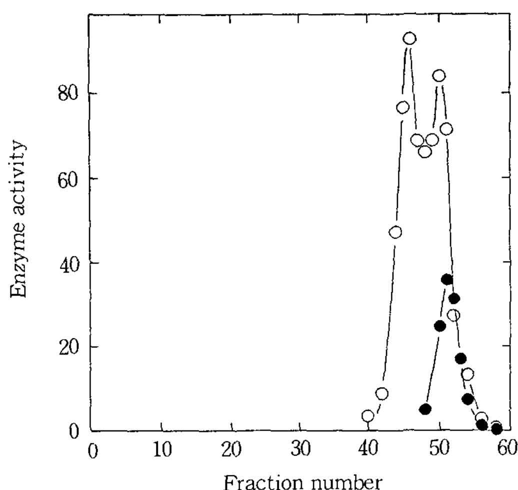

Fig. 1. Elution profile of invertase (○) and fructan exohydrolase (●) on a Sephacryl S-200 column. Enzyme activities were expressed as μmole fructose released per hr per fraction. All fractions were assayed but only those greater than zero were plotted.

ent salt concentration, but invertase was not completely separated from FEH. It is important to separate invertase from FEH because invertase can hydrolyze low molecular weight fructan. Combining the DEAE/TSK fractions containing FEH activity, and ConA-sepharose colume chromatograph separated FEH activity from residual invertase activity(Fig. 3). This final preparation showed one major band of around 66kd and three minor bands of 55, 40, and 30kd on SDS-polyacrylamide gel(Data not shown). However, because the fraction containing FEH activity peak was devoid of sucrose : sucrose fructosyltransferase (SST) and fructan : fructan fructosyltransferase (FFT) activities(Data not shown), the fraction was used to further characterize wheat FEH.

The binding characteristics of wheat FEH to ConA column indicated that this enzyme is a glycoprotein. Fructan hydrolases of barley

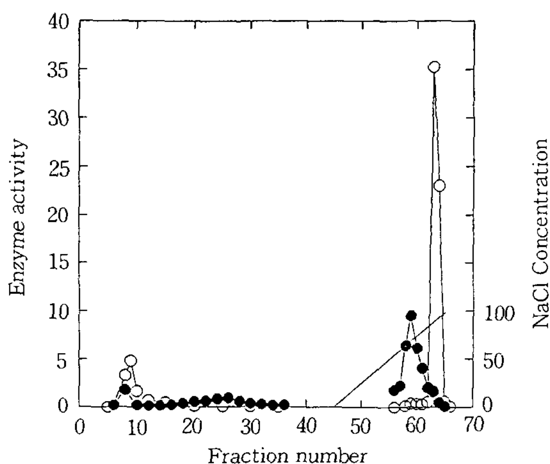

Fig. 2. Elution profile of invertase (○) and fructan exohydrolase (●) from Sephacryl S-200 fraction on a DEAE/TSK column. The bound proteins were eluted with a linear gradient of 0 to 0.1M NaCl(fractions 45 to 66).

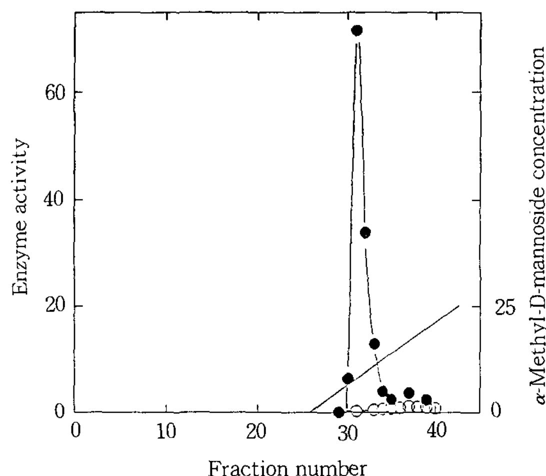

Fig. 3. Elution profile of invertase (○) and fructan exohydrolase (●) on a ConA-Sepharose column. The bound proteins were eluted with a linear gradient of 0 to 50mM α-methyl-D-mannoside(fractions 25 to 60).

Table 1. Purification of fructan exohydrolase in wheat stem and sheath

	Specific activity*	Purification fold	Recovery(%)
Crude extract	1.65	1	100
$(NH_4)_2SO_4$ (30~70%)	3.64	2.2	70.6
Sephacryl S-200	26.23	5.9	60.3
DEAE/TSK	41.75	25.3	8.0
ConA-Sepharose	587.69	356.2	6.7

Enzyme activities were assayed using Jerusalem artichoke fructan(40 mg/ml H_2O).
* μmole fructose released/h/mg protein

and *Aspergillus niger* were also shown to be glycoproteins.[8,20,22]

Following this purification sequence(Table 1) wheat FEH was purified 356-fold from wheat stems/sheaths. The final recovered activity was 6.7%. The enzyme had a specific activity of 587μmole fructose released/h/mg protein using Jerusalem artichoke fructan (DP>6) as substrate.

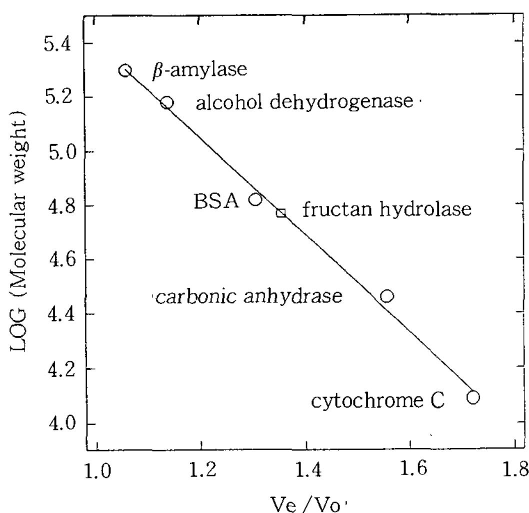

Fig. 4. Gel filtration of wheat fructan exohydrolase on a Sephacryl S-200 column. Molecular weight standards include: β-amylase(200kD), alcohol dehydrogenase(150kD), BSA(66kD), carbonic anhydrase(29kD), cytochrome C(12.4 kD). Ve is elution volume and Vo is void volume.

The molecular weight of FEH in wheat was estimated to be about 63,700(Fig. 4). FEH of barley and orchardgrass have similar molecular weight of 62,500 and 57,000, respectively.[8,23]

FEH exhibited an optimum activity at pH 5.5 using Jerusalem artichoke fructan as substrate(Fig. 5). This optimum pH was consistent with that of other enzymes isolated from vacuoles. The FEH activity decreased sharply above pH 6 and was lost completely

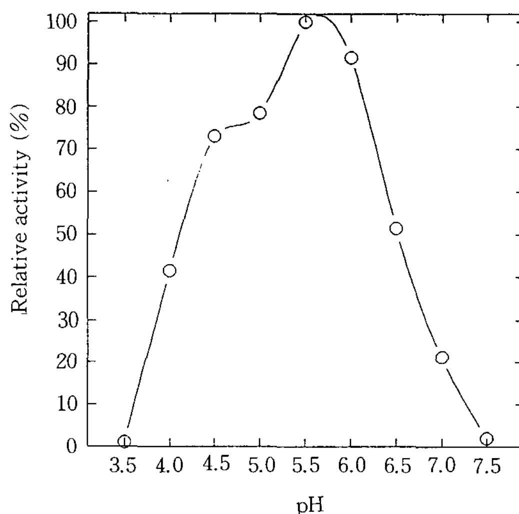

Fig. 5. Effect of pH on wheat fructan exohydrolase activity. Reaction mixtures contained one volume of Jerusalem artichoke fructan(40mg/ml), two volumes of enzyme, and two volumes of McIlvaine's phosphate/citrate buffer.

at pH 7.5. The optimum pH of wheat FEH was similar to that of barley,[8,22] orchardgrass[23] and Jerusalem artichoke.[6]

Wheat FEH activity was maximal at 30℃ and decreased rapidly above this temperature(Fig. 6). This temperature optimum was lower than those of barley FEH(35~40℃) and orchardgrass FEH(40℃). These optimum temperatures for FEH activity from different sources were unexpectedly high, particularly considering the air and soil temperatures of early spring when these grasses begin to grow and thus hydrolyze stored fructan.

Wheat FEH exhibited typical Michaelis-Menten kinetics when $\beta(2\rightarrow1)$ linked fructan oligomers were used as substrates(Fig. 7). The K_ms for each fructan were varied ranging from 10 to 37mM with the highest affinity for DP4. V_{max} tended to increase with the increasing DP. V_{max}/K_m ratio for DP4 was the greatest(5.9), indicating DP4 was the best substrate of the substrates tested. The K_m values measured in this experiment were consistent with those determined in other sources.[17] When high DP(about 30) inulin was used as substrate, K_m of FEH was about 30mM in Jerusalem artichoke,[6] 15mM in dandelion[15] and 15mM in barley.[8] Bancal et al.[1] reported that, when DP 3 to DP 5 fructans were used as substrate, barley FEH hydrolyzed the substrates with the same rates, because all these fructan oligomers contained only one terminal fructosyl unit specific to the exohydrolase, However, Edelman and Jefford[6] showed that the rate of fructan hydrolysis was largely determined by DP, increasing over the range DP 3 to about DP 6, then decreasing when substrate were above DP 6.

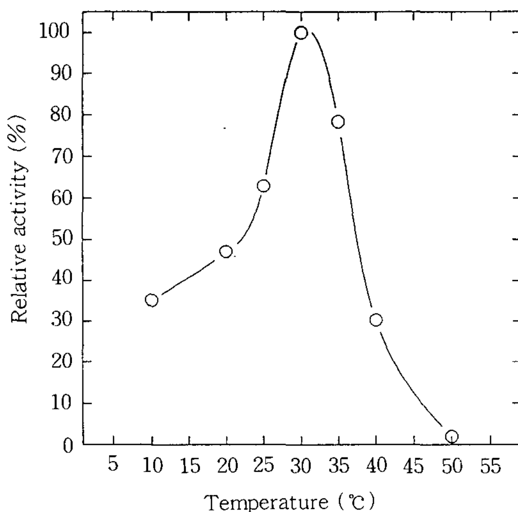

Fig. 6. Temperature effect on wheat fructan exohydrolase activity. Activities were assayed at pH 5.5 using Jerusalem artichoke fructan(40mg/ml) as substrate.

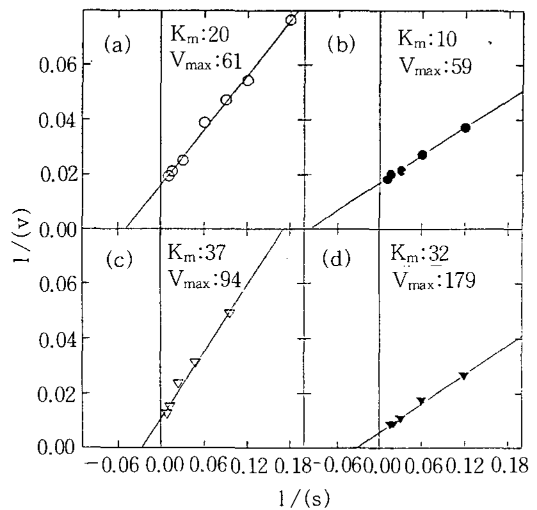

Fig. 7. Lineweaver-Burk plots of wheat fructan exohydrolase activity. The substrates were inulin-type fructans of DP 3(a), DP 4(b), DP 5(c), and DP 6 (d).

Substrate specificity of the purified wheat

Table 2. Substrate specificity of wheat fructan exohydrolase

Substrate*	Relative activity(%)
Wheat (DP>6)	100
(DP 3~6)	177.26
J. artichoke (DP>6)	244.28
Chicory	95.34
Dahlia	73.04
Timothy (DP>6)	6.11
Sucrose	2.53

* Substrate concentration of fructan was 40mg/ml and sucrose was 0.6M.

FEH was examined with fructan substrates containing 40mg fructose equivalents/ml and 0.6M sucrose(Table 2). Maximal rate of activity was obtained with Jerusalem artichoke fructan as substrate. The enzyme appeared to have a higher affinity for wheat fructan of a low degree of polymerization (DP) than high DP. Wheat FEH had a low affinity for timothy fructan which is composed of $\beta(2\rightarrow6)$ linkages. Sucrose degradation by this enzyme was negligible. These results suggested that the enzyme was specific for $\beta(2\rightarrow1)$ linked fructan. Higher affinity of wheat FEH for low DP wheat fructan was likely due to the greater terminal fructosyl concentration and the larger percent of $\beta(2\rightarrow1)$ linkages in the smaller oilgomers.[5] Because the same amount of fructan was used as substrate concentration, the low DP fructan would also have more terminal fructosyl units for exohydrolase to attack. Thus, wheat FEH had more cleavage sites in low DP fructan. According to Carpita et al.,[5] wheat contains mixed linked fructan with a higher percentage of $\beta(2\rightarrow6)$ linkage as DP increases. Two FEHs hydrolyzing $\beta(2\rightarrow1)$ linkages have been purified from inulin containing species, dandelion[15,16] and Jerusalem artichoke.[6] The FEH in phlein producing orchardgrass has a higher affinity for $\beta(2\rightarrow6)$ but can hydrolyze $\beta(2\rightarrow1)$ linkages.[23] The FEH isolated from wheat was specific for $\beta(2\rightarrow1)$ linkages, as was that isolated from barley.[8] Because barley and wheat accumulate mixed linked oligomers,[5] so a specific $\beta(2\rightarrow1)$ FEH appears unusual, an FEH with substrate preference similar to that of orchardgrass would be more likely.

Wheat $\beta(2\rightarrow1)$ FEH is involved in hydrolyzing $\beta(2\rightarrow1)$ linkages both during net synthesis and degradation. As a result of its action during net fructan accumulation, there is an increase in $\beta(2\rightarrow6)$ linkages.[1] Bancal et al.[1] suggested that all fructan structures found in wheat may begin with bifurcose, formed by a $\beta(2\rightarrow1)$ linkage synthase, an O-6 branching enzyme. Contiguous branches formed could be trimmed by the FEH specific for hydrolysis of $\beta(2\rightarrow1)$ linkage to yield $\beta(2\rightarrow6)$ linked fructans. The existence of $\beta(2\rightarrow1)$ FEH in wheat supports their hypothesis.

Wheat accumulates a large amount of fructan when exposed to 10℃ for four days. These stored fructans can be completely hydrolyzed when the blades are trimmed and transferred to 25℃ in darkness.[9] Because high DP fructans contained higher percentage of $\beta(2\rightarrow6)$ linkage than $\beta(2\rightarrow1)$ linkage, there must be another activity for cleavage of the $\beta(2\rightarrow6)$ linkage. The presence of $\beta(2\rightarrow1)$ FEH suggests that this enzyme has a different substrate specificity *in vivo* or there must be another enzyme specific for hydrolysis of $\beta(2\rightarrow6)$ linkage.

摘 要

밀의 영양체 저장탄수화물인 fructan의 대사에

관련된 fructan exohydrolase(FEH) 효소의 특성을 구명하기 위해 FEH를 밀의 줄기와 엽초에서 정제, 실험한 결과를 요약하면 다음과 같다.

1. FEH의 분자량은 63.7kD이었으며, pH 5.5와 30℃에서 최고의 활성을 보였다.
2. FEH의 K_m과 V_{max}는 fructan의 크기에 따라 각각 10~37mM, 59~179mM로 변이를 보였으며, tetrasaccharide가 본 실험에서 사용된 기질 중에서 가장 낮은 K_m과 V_{max}를 보였다.
3. 밀의 FEH는 $\beta(2\rightarrow1)$로 연결된 fructan에 대하여 기질 특이성을 보였으며 $\beta(2\rightarrow6)$ fructan과 sucrose는 분해할 수 없었다.

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