# $\beta$-Fructofuranosidase and Invertase Activity in Tall Fescue Culm Bases

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Fructosan is the primary storage polysaccharide in grass species of the Aveneae, Festuceae, and Hordeae tribes. The majority of the research on fructosan metabolism has been accomplished on the 2-1 linked inulin class of fructosan found in the Compositeae. The current research was done on the 2-6 linked levan class of fructosan stored by the Gramineae. The objective was to investigate the mode of action of $\beta$-fructofuranosidases isolated from fescue culm base tissue. The results indicate that $\beta$-fructofuranosidase, isolated from fescue culm base tissue, contained exo-action activity since polymer hydrolysis began at the fructose end of the fructosan molecule and released only fructose residues. The maximum specific activity of this enzyme, at pH 4.5 and 30 min of reaction time, was determined to be 1.23 $\mu$mol of fructose mg of protein$^{-1}$ h$^{-1}$. Invertase activity, isolated from the same tissue, was determined to be 0.88 $\mu$mol of hexose mg of protein$^{-1}$ h$^{-1}$.

Carbohydrates are the primary source of reserve energy stored in vegetative organs of biennial and perennial forage plants. DeCugnac (1931) showed that the predominant reserve polysaccharide stored in the basal vegetative parts of 15 grasses was $\alpha$-D-glucopyranosides (starch), but it was $\beta$-D-fructofuranosides (fructosan) in 23 grass species. He concluded that fructosan was the primary storage polysaccharide in grass species of the Aveneae, Festuceae, and Hordeae tribes. It has been confirmed by other researchers (Brown and Blaser, 1965; Smith, 1972; Smith and Groteleushen, 1966) that Kentucky 31 fescue (*Festuca arundinacea* Schreb.) stores surplus energy as fructosan and this stored energy is requisite for maintenance of perennial vigor.

Fructosan has the general formula, glucose–fructose–(fructose)$_n$, where G–F represents a sucrose group and $n$ is the number of additional fructosyl residues. Also, fructosan is classed as inulin, where the fructose residues are linked $2 \rightarrow 1'$ in linear chains (Rutherford and Deacon, 1972a), and as levan, where the fructose units are linked $2 \rightarrow 6'$ (Hirst, 1957). Inulin is found, most commonly, in the Compositeae and is the most fully investigated class of fructosans. Levan is common to the Gramineae and certain strains of bacteria (Evans and Bacon, 1946; Hirst, 1957) and has been investigated to a lesser extent. Levans appear to be linear molecules having a low degree of polymerization (DP). Fructosans occur in homologous series in grass tissue with the DP of the longest homologue varying with the species. Smith (1973) states that fructosans having a DP of as high as 260 have been isolated from some species of grass. Numerous researchers have studied the relationships of fructosan in tall fescue to plant growth and environmental differences (Brown and Blaser, 1965; Mackinzie and Wylam, 1957; Matches, 1969; Smith and Grotelueshen, 1966). However, little research has been accomplished on fructosan metabolism in tall fescue. Due to the significance of fructosans, as stored energy, to the perennial vigor of temperate-origin grasses, it is important to better elucidate the biochemical basis for the capacity of plants to utilize the fructosan.

Soluble protein extracts prepared from roots of dandelion (*Taraxacum officinole* Weber) contain hydrolase enzymes (Rutherford and Deacon, 1972a) that act on the 2-1 linked inulin series of fructosans (Bacon and Edelman, 1951). Hydrolysis of the fructosan may proceed either by endo-action, resulting in an essentially random hydrolysis of glycosidic linkages, or by exo-action, where the molecule is degraded in a stepwise manner from the fructose end of the polymeric chain. $\beta$-Fructofuranosidase enzymes isolated from dandelion roots are described as possessing an exo-action activity (Rutherford and Deacon, 1972b).

The objective of this research was to investigate the mode of action of $\beta$-fructofuranosidases (hydrolases) isolated from tall fescue culm base tissue.

## MATERIALS AND METHODS

A protein extract from plant tissue was used for enzyme assays. The plant tissue was obtained from 50 g of culm base material taken from a tall fescue sward maintained at medium soil fertility and clipped to a 10 cm height in Sept, 1974. Plants were removed from field plots on Jan 6, 1975 and culm bases were separated from other tissues. All culm base tissue utilized in this research was stored at 4 °C following harvest. Small quantities of tissue were macerated in 100 mM phosphate buffer (pH 7.6) containing 100 mM cysteine and 100 mM sodium diethyldithiocarbamate. A total of 75 ml of media was used to extract 50 g of culm base tissue. Initial maceration of small quantities of tissue was accomplished with a Kurzzeitbetrieb Model A-10 micro mill and the total mixture finally was mixed in a Waring blender at maximum speed for 5 min. The resulting pulp was strained through four layers of cheesecloth and the filtrate was centrifuged at 25000$g$ for 20 min. The centrifuged filtrate was concentrated to 50 ml and dialyzed against 20 mM phosphate buffer (pH 7.6) containing 5 mM cysteine and 5 mM sodium diethyldithiocarbamate, using spectrapor 1 membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.). Dialysis was continued overnight. Following dialysis, the protein was precipitated by carefully adding $(NH_4)_2SO_4$ to 95% saturation, while maintaining a pH of 7.6. After standing overnight, the precipitated protein was separated by centrifugation at 15000$g$ for 30 min, dissolved in 5 mM phosphate buffer (pH 7.6), and dialyzed overnight against 5 mM phosphate buffer (pH 7.6). The nondiffusable material was centrifuged (25000$g$) for 20 min and the clear supernatant was freeze-dried and stored or used directly for hydrolase determinations. All of the preceding work was accomplished at 4 °C. The protein concentration of the extraction mixture was quantified by the method of Lowry et al. (1951).

Levan, used for the assay of hydrolase activity, was isolated from tall fescue culm bases harvested at the same time as the culm bases utilized for protein extractions. Culm base tissue was freeze-dried and ground to pass a 40-mesh screen. Five grams of ground tissue was thrice extracted with 75 ml of 90% ethanol solution to remove

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the mono- and disaccharides (Smith, 1972). Following each extraction, the mixture was centrifuged (15000g) for 20 min and the filtrate discarded. The levan was extracted from the remaining pulp by shaker with 50 ml of $H_2O$ (Smith, 1972). Following centrifugation (15000g for 20 min), the residue was discarded. A 2-ml aliquot of filtrate was freeze-dried in 1-dram screw-top reaction vials. The resulting levan mixture was analyzed for the presence of mono- and disaccharides by the method of Phillips and Smith (1973). The dried levan in the reaction vials was dissolved in 2 ml of citrate–phosphate (0.1 M:0.2 M) buffer (pH 4.3) and the enzyme assay reaction was started by adding 2 ml of protein extract. This will be referred to as TAE (time of adding enzyme). The final pH of the reaction mixture was 4.5. Hydrolase activity on inulin was determined in 1-dram reaction vials containing 2 ml of 0.2% pyrogen-free inulin (Dahlin:Alantin) purchased from Nutritional Biochemicals Corp. in citrate–phosphate (0.1 M:0.2 M) buffer (pH 4.3).

Invertase activity of the extracted protein was accomplished in 1-dram reaction vials containing 2 ml of 0.2 M sucrose in citrate–phosphate (0.1:0.2 M) buffer (pH 7.5) and 2 ml of protein extract. All enzyme assays were conducted at 24 °C.

The mode of action (endo- and/or exo-action) of the hydrolase activity on levan and inulin was determined by the method used by Rutherford and Deacon (1972b) and using adaptations of Smith (1972) and Phillips and Smith (1973) for analyzing free fructose and glucose. At 0 and 2 h after TAE 1-ml aliquots were removed from each reaction vial for each of the following analyses: (1) free glucose and free fructose (Smith, 1972); (2) free glucose and free fructose (Smith, 1972) following acid hydrolysis (1 N HCl) (Rutherford and Deacon, 1972a); (3) free glucose and free fructose (Smith, 1972) following treatment with strong base (2 N NaOH) and acid hydrolysis (1 N HCl) (Rutherford and Deacon, 1972b); and (4) glucose, fructose, and sucrose (Phillips and Smith, 1973). In step 3, treatment with strong base always preceded acid hydrolysis. Preliminary experiments indicated negligible interference from protein extractants and buffer solution with methods utilized for quantifying carbohydrate fractions. Check solutions of buffered fructosan and sucrose solutions, without protein extract, were included in each experiment for correction due to chemical hydrolysis.

Invertase activity was quantified by measuring glucose and fructose released from sucrose using the methods of Smith (1972) and Phillips and Smith (1973) at 0 and 2 h following the introduction of protein extract into the buffered sucrose solution (TAE). The former method proved the most satisfactory. Results are reported from the colorimetric analyses of total reducing carbohydrates. Tests of each enzyme extraction were done in triplicate and the experiment was conducted twice.

## RESULTS AND DISCUSSION

The protein concentrations for the enzyme assays ranged from 1.34 to 1.47 $\mu$g/ml of protein extraction for all experiments. Gas–liquid chromatographic analyses (Phillips and Smith, 1973) of the levan isolated from tall fescue culm bases and of inulin solutions indicated that less than 4% of the carbohydrates in the reaction mixture were free glucose, fructose, and sucrose. Also, less than 3% free glucose and fructose were present in sucrose solutions prior to assaying invertase activity.

It is well known that reducing sugars undergo a complex series of degrading reactions in hot alkali, whereas nonreducing sugars are relatively stable (Rutherford and

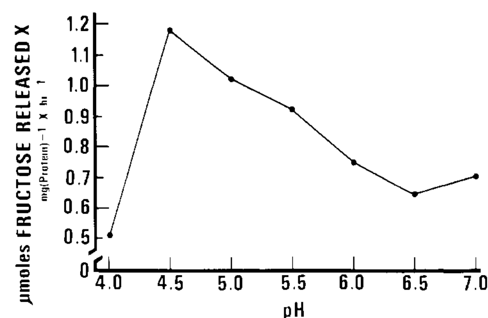


**Figure 1.** Influence of pH on $\beta$-fructofuranosidase activity.

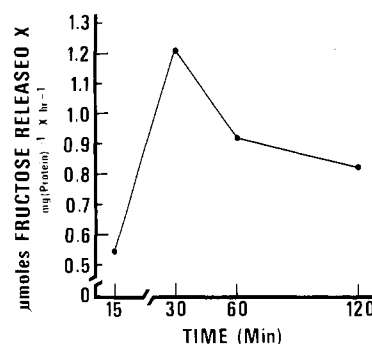


**Figure 2.** Influence of reaction time on $\beta$-fructofuranosidase activity.

Deacon, 1972b). With polysaccharides, alkaline attack commences at the reducing end and proceeds throughout the chain (Whistler and BeMiller, 1958). If hydrolase activity is an exo action beginning at the nonreducing fructose end, then the amount of fructose destroyed by basic treatment (2 N NaOH) following hydrolase action should be approximately equal to that of reducing sugar released by the enzyme. If attack is an endo action the amount of fructose destroyed should be much greater than the fructose measured following enzymatic hydrolysis. By comparing the alkali-labile fraction of the total sugar present with that released by the action of the enzyme after a limited amount of hydrolysis has occurred, it should be possible to distinguish between exo- and endo-action attack. This thesis, proposed by Rutherford and Deacon (1972b), was the basis for determining the hydrolase mode of action in this study.

The activity of $\beta$-fructofuranasidase enzymes, isolated from fescue culm bases and assayed on the levan class of fructosan, was 1.2 $\mu$mol of fructose released $\times$ mg of protein$^{-1}$ $\times$ h$^{-1}$ at a pH of 4.5 (Figure 1) and at 30 min after TAE (Figure 2). This activity would convert to approximately 0.021 $\mu$mol of fructose per g of tissue fresh weight per min and is slightly lower than the hydrolase activity reported for dandelion roots (Rutherford and Deacon, 1974). Monosaccharides in the reaction vial following enzyme activity were fructose and glucose. The free glucose content changed very little in response to enzyme activity over a 2-h enzyme hydrolysis period (Table I). The major increase in free sugar content was due to increases in fructose concentration in the reaction vial. Data in Table I indicate that the hydrolysis action on the fescue culm base levan extract released primarily fructose from the levan molecules. The total amount of free sugars released between 0 and 2 h post TAE (6.7 $\mu$mol) (Table II) was approximately the same as the increased quantity destroyed by the alkaline treatment between 0 and 2 h (8.2 $\mu$mol). Free sugars lost to alkaline treatment at both 0 and 2 h post TAE very closely approximate the free sugar

**Table I. Glucose and Fructose in Reaction Vial Containing Levan and Hydrolase Extracts from Tall Fescue Culm Base Tissue[a]**

| | μmol at time post TAE (h) | | | |
|---|---|---|---|---|
| | 0 | | 2 | |
| Treatment | Glucose | Fructose | Glucose | Fructose |
| Control | 9.8 | 7.5 | 10.0 | 14.0 |
| Alkaline–acid | 24.8 | 420.6 | 25.2 | 413.0 |
| Acid | 30.4 | 431.4 | 32.0 | 430.8 |

[a] Control = free sugars prior to alkaline (2 N NaOH) treatment and acid (1N HCl) hydrolysis; alkaline–acid = free sugars following alkaline treatment and acid hydrolysis; and acid = free sugars following acid hydrolysis.

**Table II. Free Sugars Lost in Response to Treatment in 2 N NaOH[a]**

| | μmol at time post TAE (h) | |
|---|---|---|
| | 0 | 2 |
| Control | 17.3 | 24.0 |
| Free sugars lost to alkaline treatment | 16.4 | 24.6 |

[a] Control = free sugars prior to alkaline treatment and acid hydrolysis.

content in the reaction vials at both sampling periods. This would indicate that primarily monosaccharides were being destroyed by the alkaline treatment. Therefore, the mode of action of hydrolase enzymes, in the fescue protein extract, on levan appears to be an exo action beginning at the fructose end of the polysaccharide. Attack, by the enzyme, at any internal position would allow for a much greater loss of fructose in response to the alkaline treatment. An exo-action attack at the glucose end would leave the remaining chain labile to alkali destruction. Therefore, these data indicate that the enzyme hydrolyzed the levan molecule from the fructose end, sequentially along the molecule, with concurrent release of free fructose residues until a molecule of sucrose remained. Levan isolated from fescue culm bases and used in this study maintained an average chain length of approximately 20 fructose residues based on the fructose–glucose data for the acid-hydrolyzed samples less free fructose and glucose in the levan extract (Table I).

In order to determine enzyme specificity of the hydrolase activity, research was conducted substituting inulin for the levan extract. There was no significant increase in free fructose over the 2-h assay period (Table III). Also, loss of fructose to alkali treatment did not change significantly over the same period. This would indicate that the enzyme in fescue culm bases is specific to the 2→6' fructose linkage and will not attack inulin (2→1') or that certain cofactors, present in the levan extract, are requisite for hydrolase activity.

Since the ultimate products of complete hydrolase activity on levan should be fructose and sucrose, this study was extended to determine invertase activity in fescue culm base tissue. Data (Table IV) indicate invertase activity of 0.88 μmol of hexose mg of protein$^{-1}$ h$^{-1}$ during the first 2 h post TAE. It is evident that, although this activity appears similar to that reported for hydrolase at 2 h post TAE (Figure 2), products of invertase activity are two measurable hexoses, whereas hydrolase activity released only one hexose per action. Therefore, the relative activity of the isolated invertase is approximately one-third that

**Table III. Fructose in Reaction Vial Containing Inulin following No Treatment (Control), Treatment with Alkali (2 N NaOH) and Acid Hydrolyzed (1 N HCl) (Alkaline Treatment) and Acid Hydrolysis (Acid Treatment)**

| | μmol | | |
|---|---|---|---|
| h after TAE | Control | Alkaline[a] treatment | Acid[b] treatment |
| 0 | 2.3 | 18.4 | 21.4 |
| 2 | 2.4 | 19.1 | 19.9 |

[a] Alkaline treatment connotes treatment with 2 N NaOH followed by acid hydrolysis. [b] Acid treatment connotes acid hydrolysis only.

**Table IV. Hexoses in Reaction Vial Containing Sucrose following Isolated Invertase Activity**

| | μmol | |
|---|---|---|
| h after TAE | Hexose | Hexose mg of protein$^{-1}$ h$^{-1}$ |
| 0 | 19.28 | |
| 2 | 24.30 | 0.88 |

determined for levan hydrolase.

Little is known about the functional reason for polymerization of sugars to fructosans. Many plants which contain fructosans are species which endure climatic or management induced stresses during their life cycle (Eagles, 1967; May, 1960; Sonneveld, 1962). In the past, these researchers have pointed out the importance of reserve carbohydrates to regrowth following a dormant period or harvesting. However, many questions remain to be answered. Possibly, inconsistencies that exist in attempts to relate grass regrowth and vigor to storage carbohydrates may be due to the inability of the plants to release the stored energy in a usable form. The enzymes investigated in this study are important in the chain of events leading to utilization of stored energy. However, much more information is requisite to explaining the inconsistencies in relating perennial-growth responses to environmental and management induced stresses.

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