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ENDO-β-MANNANASE IN TOMATO FRUIT

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; *endo*-β-mannanase; α-galactosidase; fruit ripening.

Abstract—Extracts of ripe tomato fruit hydrolysed locust bean galactomannan, a linear β-1,4-mannan with branches of single galactose residues. The viscosity of the reaction mixture decreased sharply relative to the release of reducing groups, indicating random hydrolysis of the mannan backbone of the polysaccharide. The *endo*-β-mannanase was purified by ion exchange chromatography and shown to be free of α-galactosidase and β-mannosidase. The M_r was estimated to be 43 000 by gel filtration. The enzyme was optimally active at pH 4.8. It was much more effective on galactomannan from locust bean than that from guar, consistent with excessive galactose branching in the latter polysaccharide. The *endo*-β-mannanase was absent in green tomato fruit but appeared as the tomatoes began to ripen and it increased during ripening, suggesting a possible role for this enzyme in the ripening process. Although *endo*-β-mannanases have been found in seeds of certain plants, this is the first report of this enzyme in other tissues.

INTRODUCTION

Galactomannan is a major component of the endosperm of seeds of a number of plants including lucerne, guar and soybean [1, 2]. This polysaccharide contains variable proportions of mannose and galactose characteristic of the source but always consists of a linear backbone of β-1,4-linked mannose with branches of single α-1,6-linked galactose residues [3]. The enzymes that develop during seed germination to utilize the galactomannans have been isolated and characterized [1, 2, 4, 5]. The degradation of a galactomannan involves endo-β-mannanase, β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22) [1, 2, 5].

In addition to the mannose in galactomannan that serves as a reserve polysaccharide in seeds, this monosaccharide occurs in other plant polysaccharides. Some of these are found in cell walls where they may have structural functions. Xyloglucan fractions containing 1,4-linked mannosyl residues have been isolated from cabbage [6] and tobacco [7]. Galactoglucomannans have been identified in both the extracellular polysaccharides and the cell walls of suspension-cultured tobacco cells [8]. Jarvis *et al.* [9] found that a major hemicellulose in the cell walls of potato tubers was a mannan or glucomannan. Mannose is an important component of the cell walls from fruits such as apples [10] and tomatoes [11]. Stevens and Selvedran [12] obtained a polysaccharide fraction from depectinated apple cell walls that contained a very high proportion of 1,4-linked mannosyl residues. Despite the widespread occurrence of mannose-containing polysaccharides in cell walls, there is no information on enzymes that can degrade these components. This paper describes an enzyme from ripe tomato fruit that hydrolyses β-1,4-mannans.

RESULTS AND DISCUSSION

Detection of mannosyl-cleaving enzymes in tomato fruit

Extracts of ripe tomatoes contained an abundance of α-mannosidase according to an assay with the substrate *p*-nitrophenyl-α-mannoside. This enzyme was readily extracted from the tissue with water, indicating that it was not associated with the cell walls. Enzymatic activity was not detected with *p*-nitrophenyl-β-mannoside nor with yeast mannan which consists of α-1,2- and α-1,6-linkages. Extracts of ripe tomatoes were effective in hydrolysing locust bean galactomannan, and a rapid decrease in viscosity of the polysaccharide (data not shown) suggested that the enzyme was a mannanase. As McCleary and Matherson [2] pointed out, the removal of the galactose side chains from the polysaccharide by α-galactosidase would have caused little change in viscosity until the galactose were reduced to 12% when the polysaccharide becomes insoluble. Thus, the enzyme in tomato extracts appeared to be attacking the mannan backbone of the polysaccharide.

Purification of enzyme

The procedure developed earlier for the extraction of polygalacturonase [13] proved to be a convenient method for extracting the mannanase. It involves washing the cell wall fragments with water at pH 3 to remove soluble proteins, including most of the α-galactosidases. The washed fraction is then extracted with water at pH 1.6 to solubilize the cell wall enzymes.

The crude extract contained a low level of α-galactosidase. This enzyme slowly hydrolyses the galactose side-chains in galactomannan [2] and this can influence

the assay for mannanase in two ways. Debranching of the substrate molecule would increase the susceptibility of the mannan backbone to mannanase action [2], and the release of galactose would interfere in the reductometric assay for mannanase. Thus, it was important to develop a purification procedure that removed the two isoenzymes of α-galactosidases [14] from the mannanase.

On chromatography of the crude extract on DEAE-Sephadex A-50, the mannanase eluted on the shoulder of a large protein peak containing polygalacturonase along with a trace of α-galactosidase I (Fig. 1). α-Galactosidase II eluted in fractions 62–66 and was therefore easily removed by this step. The mannanase in fractions 17–23 was then chromatographed on S-Sepharose (Fig. 2). This step removed both α-galactosidase I (fractions 12–15) and polygalacturonase (fractions 35–39). The mannanase in fractions 25–28 was finally purified on a Mono S column in a fast protein LC system (Fig. 3).

A summary of the purification of *endo*-β-mannanase is presented in Table 1. The enzyme solution contained only one protein band according to isoelectric focusing and silver staining. It was free of α- and β-galactosidases, α- and β-glucosidases, and α- and β-mannosidases as well as polygalacturonase.

Mechanism of mannanase action

The purified enzyme rapidly decreased the viscosity of 0.25% locust bean gum with a slow concomitant release of reducing groups. A large change in viscosity relative to a small change in reducing groups is consistent with random cleavage of the mannan backbone of the polysaccharide. This was confirmed by examining the products of the reaction. Gel filtration on Sephadex G-100 showed that the products decreased in size progressively with reaction time, but the reaction did not proceed beyond a limit product estimated to have a M_r of 2 500. The size of the limit product was confirmed by chromatography on Bio-Gel P-6. The limit product was found to consist of galactose and mannose by hydrolysis with TFA and analysis of the monosaccharides [15]. Free galactose and mannose were not detected in the enzyme reaction sol-

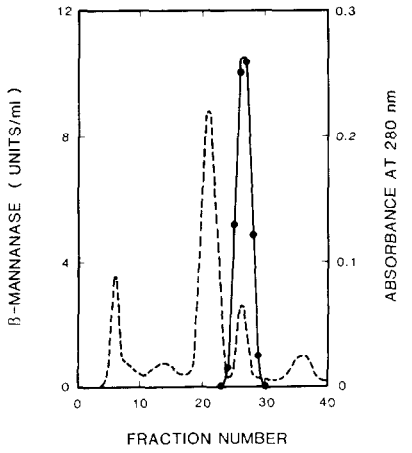

Fig. 2. Chromatography of DEAE-Sephadex A-50 fraction on S-Sepharose. ●——●, *endo*-β-Mannanase; - - - - -, A_{280}.

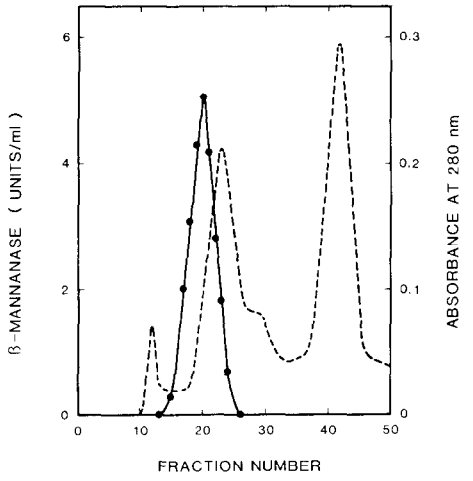

Fig. 1. Chromatography of crude tomato extract on DEAE-Sephadex A-50. ●——●, *endo*-β-Mannanase; - - - - -, A_{280}.

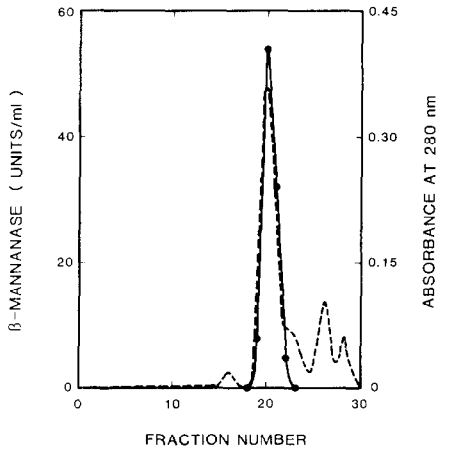

Fig. 3. Chromatography of S-Sepharose fraction on Mono S. ●——●, *endo*-β-Mannanase; - - - - -, A_{280}.

Table 1. Summary of purification of *endo*-β-mannanase from tomato fruit

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	25	46	730	16	—
DEAE-Sephadex A-50	5	13	610	47	83
S-Sepharose	2	2.2	490	223	67
Mono S	2	1.4	380	271	52

ution, consistent with the absence of α-galactosidase and β-mannosidase in the purified enzyme.

Guar galactomannan was also hydrolysed by the tomato mannanase but at only a twentieth of the rate for the locust bean polysaccharide in terms of the release of reducing groups. The enzyme decreased the viscosity of the reaction mixture markedly, indicating that the backbone of guar galactomannan was hydrolysed. McCleary and Matheson [2] found that the hydrolysis of galactomannan by mannanase was strongly influenced by the prevalence of galactose side chains on the mannan backbone. The rate of hydrolysis was high when galactose substitution was low but it dropped abruptly when the percentage of galactose exceeded 25 to 30. They reported that guar galactomannan contains 33% galactose which is considerably higher than that for locust bean gum [2]. Degradation of the guar polysaccharide by tomato mannanase was enhanced somewhat by the addition of either tomato α-galactosidase I or II. As noted earlier [14], the tomato galactosidases are rather ineffective on galactomannan, requiring long reaction periods to remove appreciable amounts of the galactose.

Other properties of enzyme

The tomato endo-β-mannanase was optimally active at pH 4.5 to 5, in agreement with pH optima reported for *endo*-β-mannanases from other sources [2, 5]. The reaction rate was not affected by the nature of the buffer or by salts in general. The enzyme could be stored at −20° for several months without appreciable loss of activity. It was relatively heat labile, with complete inactivation after 5 min. at 60°. The M_r was estimated to be 43 000 by gel filtration on a column of Sephadex G-100 calibrated with cytochrome C, carbonic anhydrase, ovalbumin and bovine serum albumin. The p*I* of the enzyme was estimated to be 9.3 by isoelectric focusing.

Changes in endo-β-*mannanase in tomatoes during ripening*

The fruit of two tomato cultivars (Big Boy and Better Boy) each at four stages of ripeness (mature green, turning, medium ripe and ripe) were analysed for *endo*-β-mannanase. Extracts were prepared from 1 kg of pericarp tissue from each sample of fruit and purified through the S-Sepharose step to remove the α-galactosidases. *endo*-β-Mannanase was not detected in the extract from green Big Boy tomatoes and it was extremely low in that from green Better Boy (Table 2). Fruit from both cultivars beginning to develop red colour contained substantial amounts of the enzyme. The fruit developed more *endo*-β-mannanase on further ripening, but the activity appeared to level off before the fully ripe stage.

The appearance of endo-β-mannanase in ripening tomatoes resembles the changes in polygalacturonase [13, 16]. A difference in the formation of the two enzymes is that endo-β-mannanase increases more markedly during the initial stages of tomato ripening that does polygalacturonase. Polygalacturonase increases gradually during ripening and is generally highest in overripe tomatoes [13, 16]. While polygalacturonase has been implicated in fruit softening accompanying ripening, the role of *endo*-β-mannanase in this process remains unknown. Mannans have been found in cell walls [6, 9], but further studies are needed to determine if these polysaccharides are degraded by the mannanase during ripening.

Table 2. Endo-β-mannanase in tomatoes during ripening

Stage of ripeness	Big Boy	Better Boy
	Units *endo*-β-mannanase/g tissue	
Green mature	0	0.01
Turning	0.29	0.23
Medium ripe	0.43	0.48
Ripe	0.50	0.49

EXPERIMENTAL

Enzyme extraction and purification. One kg of pericarp tissue from ripe tomatoes was homogenized in 1 l of H_2O. The homogenate was adjusted to pH 3 with dil. HCl, stirred for 15 min and centrifuged. The insol. fr. was washed with 1500 ml of H_2O at pH 3 and collected by centrifugation. The enzyme was then extd by suspending the pellet in 1500 ml of H_2O at pH 1.6. After stirring for 1 hr, the supernatant soln obtained by centrifugation was adjusted to 0.15 M NaCl and pH 6 and concd to 25 ml by ultrafiltration using a PM-10 membrane (Amicon Corp). The soln was applied to a 5 × 70 cm column of DEAE-Sephadex A-50 adjusted to pH 6 and equilibrated with 0.15 M NaCl; the column was eluted with 0.15 M NaCl. The frs were assayed viscometrically and reductometrically with locust bean galactomannan as substrate. Frs containing activity were pooled, ultrafiltered to 5 ml and dialysed against 0.02 M NaOAc, pH 5.5.

The enzyme soln was then applied to a 2.5 × 14 cm column of S-Sepharose equilibrated with 0.02 M NaOAc, pH 5.5. Elution was conducted with 500 ml of a linear gradient of 0–0.5 M NaCl in 0.02 M NaOAc, pH 5.5. The frs containing *endo*-β-mannanase were pooled, concd to 2 ml and dialysed against 0.02 M NaOAc, pH 5. The soln was finally chromatographed on a Mono S column in a fast protein LC system (Pharmacia). Elution was conducted with 30 ml of a linear gradient of 0–0.3 M NaCl in 0.02 M acetate, pH 5. Frs containing *endo*-β-mannanase were pooled, ultrafiltered to 1 ml and dialysed against 0.02 M NaCl.

Assay for endo-β-mannanase. The enzyme was assayed by adding 0.1 ml of enzyme soln to a reaction mixt. consisting of 0.3 ml 0.05 M NaCl, 0.1 ml 0.2 M NaOAc, pH 4.8, and 0.5 ml of 0.5% locust bean gum in H_2O. After 1 hr at 37°, the solns were analysed for reducing groups by the cyanoacetamide reagent [17]. The formation of reducing groups was linear with time. A unit of *endo*-β-mannanase is defined as that amount which catalyses the release of 1 μmol of reducing groups under these conditions.

The mannanase was also assayed viscometrically. The reaction mixt. was increased 5-fold to 5 ml and placed in an Ostwald viscometer immersed in H_2O at 37°. The viscosity was measured at regular intervals for 1 hr.

Electrophoresis. The purified enzyme was evaluated for purity by isoelectric focusing on Phast Gel 1EF 3-9 with silver staining using a Phast System (Pharmacia).

Other methods. Glycosidases were assayed by measuring the release of nitrophenol from *p*-nitrophenylglycosides at pH 4.5 [14]. Protein was measured by the method of ref. [18] and in chromatographic frs by A_{280}. Carbohydrates were determined by the PhOH method [19].

Substrates. Locust bean and guar gums and the *p*-nitrophenylglycosides were obtained from Sigma. Locust bean gum was described as a straight chain polymer of mannose with one galactose branch every fourth mannose, M_r *ca* 310 000.

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