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ENZYMIC ACTIVITIES RESPONSIBLE FOR XYLOGLUCAN DEPOLYMERIZATION IN EXTRACTS OF DEVELOPING TOMATO FRUIT

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

AHMED FAIK, DARRELL DESVEAUX and GORDON MACLACHLAN*

Department of Biology, McGill University, 1205 Dr. Penfield Avenue, Montreal, Que., Canada H3A 1B1

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Key Word Index— *Lycopersicon esculentum* fruit; Solonaceae; endo-1,4-β-glucanase (cellulase); xyloglucan endotransglycosylase (XET); xyloglucan oligosaccharide subunits; xyloglucanase (XGase).

Abstract—The capacity of salt-soluble enzymic extracts of young green tomato fruit to reduce the viscosity and molecular size of tamarind xyloglucan (XG) in solution is stimulated markedly by the addition of XG subunit oligosaccharides. This is not due to end-product activation of an endo-1,4-β-glucanase or XGase, for the following reasons: non-specific endo-1,4-β-glucanase (carboxymethylcellulase) activities in developing tomato fruit were recovered from buffer and detergent-extracts but were barely detectable in subsequent extracts with 1 M NaCl, where XG-depolymerizing activity predominated. Salt-soluble extracts alone were totally unable to degrade high M_r XG from tamarind seed in the absence of fragments of low M_r XG or oligosaccharide subunits. The degradation catalysed by tomato salt extract plus tamarind XG and XG oligosaccharides failed to proceed to completion or generate low M_r end products, as happens in the presence of cellulase. The reactions progressed with no detectable net increase in total reducing power of the reaction mixtures, but were accompanied by condensation of XG cleavage fragments with XG [^{14}C]oligosaccharide, as expected from XG endotransglycosylase (XET) activity. The activity leading to the depolymerization of XG, as assayed viscometrically in the presence of XG oligosaccharide, co-chromatographed in various media in the same fractions as XET activity, as assayed radiometrically. It is concluded that XET activity in salt extracts of young green tomato is sufficient by itself to explain the observed capacity of extracts to depolymerize tamarind XG. *In vivo*, 1,4-β-glucanase or XGase activity would also be required to generate the low M_r XG acceptors needed for transglycosylase to catalyse a net depolymerization of XG.

INTRODUCTION

The rate at which enzymic extracts of tomato fruit depolymerize chains of xyloglucan (XG), as assayed by measuring loss of viscosity of XG solutions, is stimulated by the addition of oligosaccharide subunits of XG [1, 2]. Theoretically, this could be explained either by hydrolysis with an endo-1,4-β-glucanase (E.C. 3.2.1.4) which is activated by XG oligosaccharides (the end-product of the reaction), or by non-hydrolytic cleavage with a XG-specific endo-transglycosylase (XET) using XG oligosaccharide as acceptor. The term xyloglucanase (XGase) has been used to refer to either β-glucanase or XET when they catalyse XG hydrolysis in the absence of added XG oligosaccharides. Probably most endo-1,4-β-glucanases are able to hydrolyse XG to some extent although, to our knowledge, none has been shown unequivocally to be stimulated allosterically by XG subunits. In contrast, XET was first purified from extracts of germinating nasturtium seeds by assaying its activity as a specific XGase in the absence of added XG oligosaccharides [3]. Subsequent studies [4, 5] showed that this enzyme catalysed a more rapid loss of XG viscosity in the presence of added XG subunits by acting as a transglycosylase with the subunits as acceptors. The present study employs a variety of biochemical techniques to examine the breakdown of XG in the presence of extracts of tomato fruit, plus or minus added XG oligosaccharides. We have used crude rather than purified extracts in this study, delib-

* Author to whom correspondence should be sent.

erately, in order to establish the range of XG-depolymerizing enzymes in tomato fruit and to assign responsibility for the decrease in XG chain length that these enzymes catalyse *in vitro*.

Enhanced XG depolymerization by plant enzyme extracts in the presence of XG oligosaccharides was first observed in experiments with partially purified endo-1,4-β-glucanase activity from *Pisum* epicotyls [6] and *Phaseolus* leaves [7] acting on solutions of high M_r seed XG. The stimulation of XG viscosity loss was specific to XG oligosaccharides. It did not occur when carboxymethylcellulose (CMC) was the substrate or when *Trichoderma* cellulase was the catalyst. The results were interpreted at the time [6, 7], as activation of the β-glucanase (XGase) by XG oligosaccharides. Shortly thereafter, however, XET was discovered and shown to be present in such extracts [4, 5, 8–10] where it could have evoked or contributed to loss of XG viscosity by catalyzing XG cleavage by transglycosylation. Similar experiments with extracts of tomato fruit [1, 2] demonstrated the presence of at least three CMCases which could act as XGases, plus XG depolymerizing enzyme activity that could not hydrolyse CMC but depended on added oligosaccharides. Some of the added oligosaccharides were incorporated into high M_r XG even as the net XG chain length declined [2], indicating the presence of XET. It was evident from the biochemical results that multiple enzymes able to degrade XG were distinguishable in tomato fruit and other growing or senescing plant tissue by differences in pH optima, substrate specificity, developmental characteristics, solubility and chromatographic mobility.

Such multiplicity has now been confirmed and extended by molecular studies that have established the existence of a multigene family in plants coding for what has been called "xyloglucan-related proteins" [11]. There are at least six structurally distinct 1,4-β-glucanase genes in tomato [12] which possess sequences targetting them for secretion and tissue-specific expression, plus three β-glucanases which contain hydrophobic sequences typical of integral membrane proteins [13]. These enzymes are expressed especially in growing tissues, including fruits. There is also evidence for a multigene family of divergent tomato XET genes that exhibit sequence homology to nasturtium and other XETs but not to the 1,4-β-glucanases [14–16]. It is now possible to conduct genetic transformation tests to alter expression of these various genes and help determine their functions and possible interactions.

A prominent question that remains to be answered by biochemical experimentation is whether it is necessary to invoke an oligosaccharide-activated XGase to explain the XG viscosity loss catalysed by tomato extract, or whether it can be explained entirely by XET activity. This is the subject of this paper. Five methods have been used in present tests for detecting XG depolymerization. (a) Viscosity loss of XG solutions measures the initial rate of degradation of mainly the longest chains of polysaccharide [17]. Tamarind seed XG from Megazyme has an average chain length $> 10^6$ Da [18] and, in our experience, viscous XG solutions become as fluid as water when their chain length is reduced below about 10^5 Da dextran equivalents. Thus, any enzyme activity that catalyses a net reduction of XG chain length, either by hydrolysis or transglycosylation, can be detected by assaying for viscosity loss of high M_r XG. (b) Radiometric procedures measure the transglycosylation of cleavage fragments of XG chains to XG oligosaccharide labeled either with [^{3}H] [8] or [^{14}C] [2, 4, 19] and are a specific assay for XET. (c) A specific colorimetric assay for XG [20] depends on the production of a blue–green iodine complex with XG that has a chain length above about 2×10^4 Da [21]. Depolymerization of long chains of XG results in loss of iodine color in a manner that correlates linearly with loss of viscosity [22]. (d) Reductometric methods measure a net generation of reducing chain ends that occurs during depolymerization of XG by hydrolase action but not by transglycosylation, since the latter forms a new linkage after each cleavage with no net change in reducing power. Finally, (e) XG depolymerization may be visualized directly by gel filtration chromatography as a fall in chain length distribution in profiles eluted from columns (e.g. Refs. [2, 5, 6, 18]). Present results are summarized in *Plant Physiol. Suppl.*, 1997, **114**, 151.

RESULTS AND DISCUSSION

Solubility and levels of XG-depolymerizing enzyme activities in extracts of tomato fruits

Table 1 shows the yields on a fresh weight basis of enzyme activities able to cleave XG (XET) and those able to reduce XG viscosity in extracts of young green tomato fruit (12 g fr wt average), compared to yields from mature red fruit (150 g fr wt). XET activity was assayed by the [^{14}C] radiometric procedure and XG depolymerization by the initial rate of loss of viscosity of solutions of tamarind XG (0.5%) in the presence of added XG oligosaccharide (0.02% w/v). Much of the tissue protein (60%) was extracted, presumably from the fruit apoplasm or cytoplasm, by an initial homogenization in dilute buffer, but only a small part (10–15%) of the total enzyme activity, assayed radiometrically or viscometrically, was recovered in this fraction. Subsequent extraction of buffer-insoluble residues with detergent solubilized a further 30% of total protein. Almost none of the enzyme activity (<2%) from young growing fruit was in this membrane fraction, although about one-quarter of the activity in mature ripening fruit was solubilized. Only a minor part (10%) of total protein remained to be extracted by high salt solutions from the detergent-insoluble residues. But this fraction contained most of the recovered enzyme activity. Total activities per unit fresh weight and specific activities were much higher

Table 1. Recovery of XG-metabolizing enzyme activities* in extracts of tomato fruit obtained by sequential homogenization in dilute buffer (20 mM phosphate, pH 6), detergent (1% triton X-100) and salt (1 M NaCl)

Tomato	Extract	Protein (μg/g fr wt)	XG-metabolizing activity* (units/g fr wt)	
			radiometric	viscometric
Growing (green)	buffer	1280	46	474
	detergent	660	5	62
	salt	190	255	3530
Ripening (red)	buffer	720	3	38
	detergent	330	12	73
	salt	133	29	178

*Reaction conditions and the definition of units are described in Section 3. Note that it is not meaningful to compare absolute values for units between the two assays as defined here.

Table 2. Recovery of non-specific endo-1,4-β-glucanase (cellulase) activity, using carboxymethyl-cellulose (CMC) as substrate, in sequential extracts of tomato fruit*

Tomato	Extract	CMCase activity* (units/g fr wt)	
		pH 5	pH 7
Growing (green)	buffer	37	135
	detergent	93	119
	salt	2	5
Ripening (red)	buffer	13	12
	detergent	3	5
	salt	4	11

*Extracts prepared as in Table 1 were used to measure the initial rate of depolymerization of 0.5% CMC solutions by viscosity loss at pH 5 and 7, the optima of the CMCase activities in tomato [1]. Activities were the same in the presence or absence of added XG oligosaccharides.

(10–20 fold) in salt extracts of green than red fruit, regardless of the assay procedure.

The distribution of non-specific endo-1,4-β-glucanase activities in the extracts prepared above is shown in (Table 2). CMCase activities per unit fresh weight were recovered almost entirely in buffer and detergent extracts. Total and specific CMCase activities were higher in green than in red fruit and probably accounted for at least part of the XG degradation that was assayed by viscosity loss in these extracts (Table 1). Less than 2% of the total yield of CMCase activity was salt-soluble.

It is concluded that growing green tomato fruit is a much richer source than red ripening fruit of enzymic activity on a fresh weight or protein basis that can degrade 1,4-β-glucan or XG. The activities vs CMC and XG were clearly due to enzymes with different solubilities and, by implication, different intracellular distributions. The observation that cellulase activities assayed viscometrically (Table 2) were mainly buffer- and detergent-soluble (Table 2) is consistent with the gene sequence data [12, 13] which indicated that these enzymes were either secreted or membrane-bound. But the main XG-degrading activities were dependent on added XG oligosaccharide and were tightly bound to a non-membranous insoluble part of the cell, presumably the wall, by ionic linkages that could be broken by high salt concentrations. There was no ambiguity in the evidence that salt-soluble extracts of green tomato fruit contained XET activity, as indicated by incorporation of [^{14}C]XG oligosaccharides into 67% ethanol-insoluble XG (Table 1). Such extracts have also been reported [16] to incorporate label from tritiated XG oligosaccharides into XG fragments that bind to cellulose filters, i.e. an assay for XET devised by Fry *et al.* [8, 9]. This activity was purified and sequenced [16] and found to correspond closely to the XET isoform that had been cloned earlier from *Vigna* [14]. The question at issue here is whether the XET activity in these salt extracts is

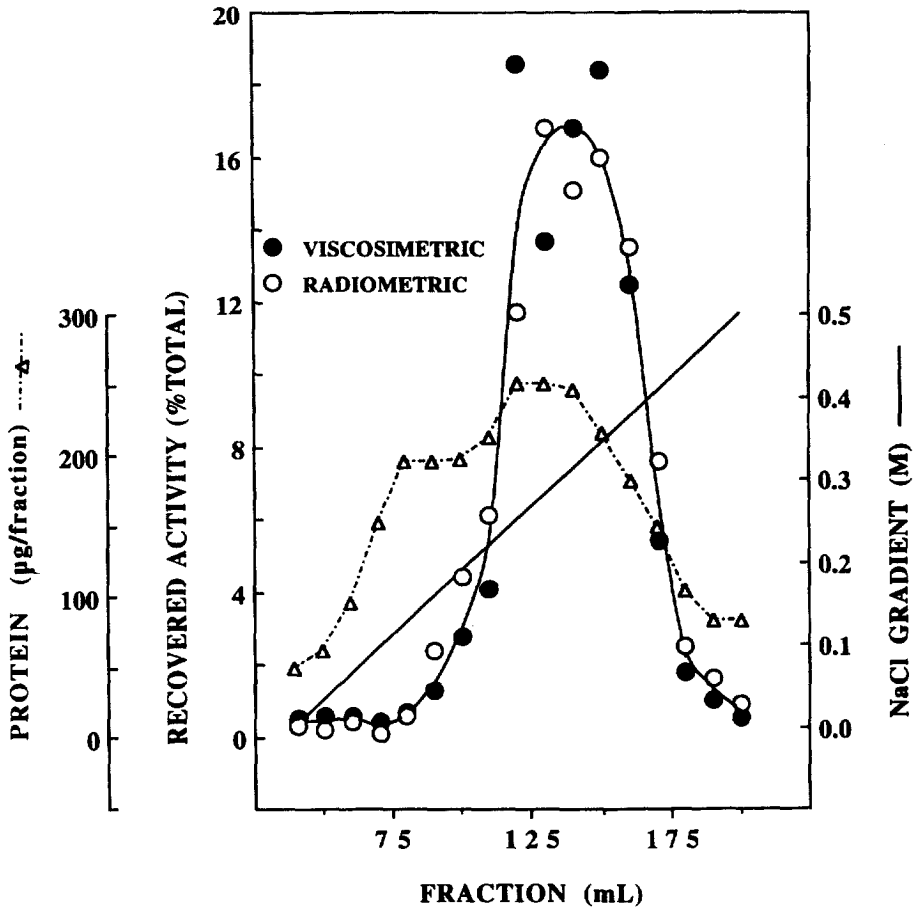

Fig. 1. Ion exchange chromatography of protein (-△-) and XG-metabolizing enzyme activity of salt-soluble extracts of growing green tomato fruit, as assayed viscometrically for depolymerization (– ● –) or radiometrically for transglycosylation (– ○ –). Fractions were eluted by a linear NaCl gradient (0–0.5 M) from a column (500 ml) of CM-TrisAcryl M.

sufficient to explain the depolymerization indicated by the viscometric assay of these same extracts, or whether additional oligosaccharide-activated glucanase/XGase activity has to be invoked.

Fractionation of XG-degrading activities as detected by radiometric (XET) and viscometric assays

Salt-soluble extract of growing green tomato fruit was dialysed vs 0.05% NaCl and fractionated by cation exchange chromatography on a column of CM Tris–Acryl by elution with a linear NaCl gradient. About one-third of the protein in this extract eluted at relatively low ionic strength (below 0.2 M NaCl) before any significant XG-metabolizing enzymic activity emerged from the column (Fig. 1). The rest of the protein and the activity as assayed viscometrically or radiometrically eluted between 0.2 and 0.4 M NaCl. The profiles for the two methods of measuring activity were indistinguishable.

The extract was fractionated by affinity chromatography on a column of Concanavalin A-Sepharose by elution with a linear gradient of α-methylmannoside. Most of the protein passed through the column before the XG-metabolising activity, which eluted between 0.5 and 1.5 M α-methylmannoside (Fig. 2). The specific activity of the peak of eluted activities for both assay procedures was increased about 15-fold over initial unfractionated activity values. Likewise, both activities bound to columns of pea XG:cellulose macromolecular complexes (cell wall ghosts [21]), but about 30% was eluted by cold 1 M NaCl and the rest by autodigestion at room temperature in the presence of XG oligosaccharides (data not shown). In summary, we were unable to distinguish between XET activity as assayed radiometrically vs XG depolymerization as assayed viscometrically, using fractionation techniques that separated proteins according to charge or binding capacity to lectin or XG.

Reaction progress: Losses in viscosity and iodine color

Salt-soluble enzyme extract from green tomatoes plus XG oligosaccharides evoked a rapid initial rate of loss of viscosity of solutions of tamarind XG which,

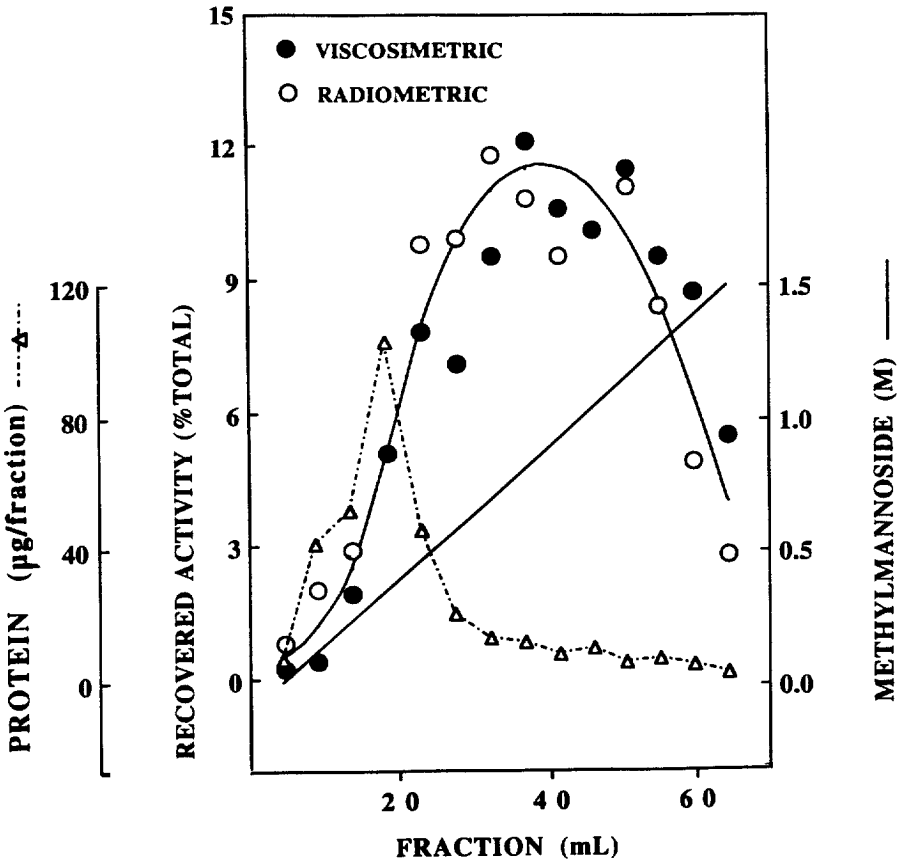

Fig. 2. Lectin affinity chromatography of protein (-△-) and XG-metabolizing enzyme activity of salt-soluble green tomato extract, as assayed viscometrically (-●-) or radiometrically (-○-), in fractions eluted by a linear gradient (0–1.5 M) of α-methylmannoside from a column (15 ml) of Con A-Sepharose.

in time, reached an asymptotic equilibrium at 10 to 20% of the initial viscosity (Fig. 3). The blue–green color of the iodine:XG complex declined in parallel with viscosity but not as rapidly (Fig. 3). The iodine color, like viscosity, was never extinguished completely, even after three days of incubation, which implies that some long chains of XG remained intact in the reaction medium. Limited depolymerization was not due to denaturation of the enzyme(s) responsible, since the addition of fresh tamarind XG immediately increased the viscosity which then declined rapidly [Fig. 3(B)], indicating that enzyme was still active when the reaction mixture was presented with an acceptable XG substrate.

Unlike the enzymes in tomato extract, *Trichoderma* cellulase is capable of degrading tamarind XG completely to its subunit oligosaccharides [18, 19]. The progress of depolymerization (Fig. 4) differs from that shown by tomato extract (Fig. 3) in that the decay in iodine color is linear with time, until it approaches close to zero (see also Table 3), and viscosity eventually reaches the fluidity of water. This would be expected from an endohydrolase with a totally random attack on XG chains. If the trace of β-glucanase activity recovered in salt-soluble extract of tomato (Table 2) also hydrolysed XG at random, it cannot have contributed significantly to the limited depolymerization of XG which the extract catalyses (Table 1).

Tomato salt extract showed only a limited capacity to reduce the viscosity of tamarind XG solution when XG oligosaccharides were omitted from the reaction mixture (Fig. 5, curves B vs A) (see also Ref. [2]). The addition of oligosaccharide to the *incomplete* reaction mixture at day one, when the slow loss of viscosity had nearly ceased at about 85% of initial values (Fig. 5, curve B), resulted in an immediate and relatively rapid viscosity loss. Evidently, the enzyme responsible for depolymerization was still potentially active after one day in the reaction mixture and the substrate XG was still accessible, but the addition of oligosaccharide was necessary for continued reaction to take place. In contrast, the addition of fresh oligosaccharide to the *complete*, reaction mixture at day one, when viscosity had fallen to about 30% of initial values and was approaching asymptotic stasis (Fig. 5, curve A), failed to lower the viscosity further. Evidently depletion of oligosaccharide was not a limiting factor in this system.

In earlier tests with extracts of tomato fruit [2] and

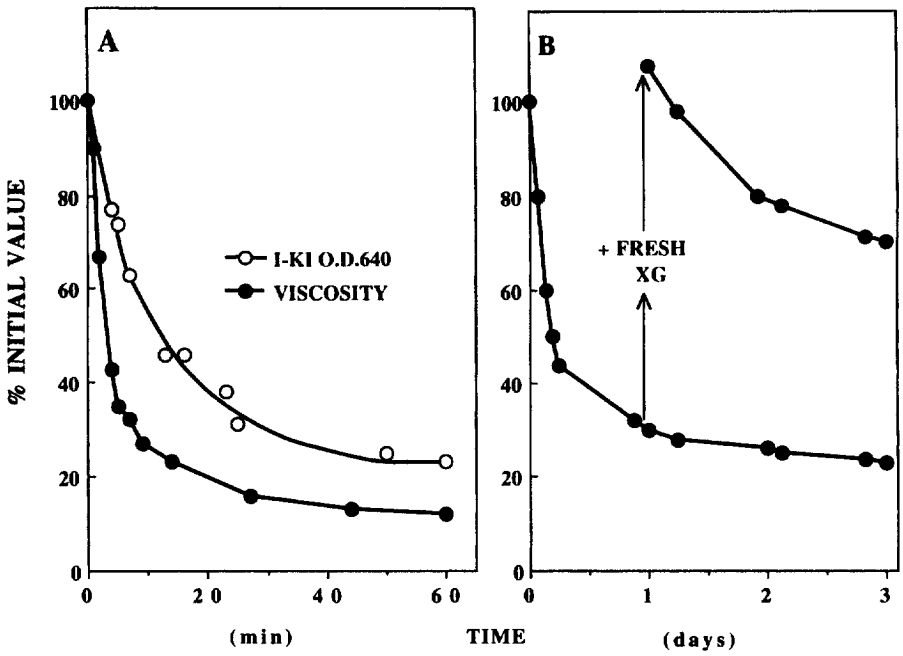

Fig. 3. Progress curves for loss in viscosity (– ● –) and color (OD_{640}) of the XG:iodine complex (– ○ –) by 0.5% tamarind XG solutions in the presence of 0.02% XG oligosaccharide and salt extract of green tomato fruit. (A) Rapid depolymerization of XG in standard reaction mixtures (1.0 ml) containing 50 μg salt-soluble tomato protein (950 units of viscometric activity, see Table 1). (B) Slow depolymerization in presence of 1 μg tomato protein. At 1 day, an extra 0.5 ml of 1% tamarind XG was added to a duplicate reaction mixture.

nasturtium cotyledons [4, 5], the comparatively slow loss of viscosity observed in the absence of added XG oligosaccharide (as in Fig. 5, curve B) was attributed to XGase activity or to XET acting as a weak hydrolase. However, hydrolase activity would have been expected to continue to reduce the viscosity at a low rate rather than ceasing prematurely at about 85% of initial values. Indeed, plant cellulases generate reducing chain ends in products formed well after the viscosity falls to the fluidity of water [12]. Another possible explanation is that shorter chain lengths of XG in reaction mixtures, which are normally present in natural populations of the polysaccharides, acted as acceptors for cleavage products of the longest chains. Since only long chains contribute to viscosity, the result would be viscosity loss until the short chains were depleted and resumption of viscosity loss if oligosaccharides were then added, as observed (Fig. 5, curve B). Accordingly, standard reaction mixtures were prepared from tamarind XG containing only chains longer than the equivalent of dextran 10^6 Da, i.e. the fraction that eluted in the void volume after Sepharose CL-6B chromatography, see Fig. 6 and Refs. [4, 18, 19]. Tomato extracts showed no capacity to reduce the viscosity of this high M_r XG unless low M_r XG fragments or XG oligosaccharides were added (Fig. 5, curve C).

It is concluded that salt extracts of tomato were completely dependent on the presence of relatively short fragments of XG before they could depolymerize long XG chains. Evidently, the XET activity in these tomato preparations resembles the XET in bean (*Vigna*) [10, 11, 14, 16] which cannot act as a hydrolase (XGase) in the absence of short chain XG acceptors. This distinguishes it from nasturtium XET which alone, in the absence of added oligosaccharides can indeed slowly depolymerize the longest chains of tamarind XG that were used in the above test (data not shown), by acting as a XGase [3–5].

Size of products

About 80% of the commercial tamarind XG used in this study fractionated on Sepharose CL-6B in the void volume (V_o), coeluting with dextrans possessing a $M_r > 10^6$ Da (Fig. 6, top panel). The rest eluted with smaller dextrans with a size down to about 7×10^4 Da. The [^{14}C] XG oligosaccharides that were used for the assay of XET, as detected either by assaying for label or carbohydrate, eluted near the V_t of this column. When standard reaction mixtures containing these components were incubated with 1 μg protein from green tomato salt extract, viscosity fell to 30% of the initial value by 1 day and to 25% by 3 days [Fig. 3(B)]. The size distribution of the XG (Fig. 6) declined correspondingly, leaving only about 25% in the void volume after 1 day and 20% after 3 days. The partially depolymerized XG was included in the column over a wide size range down to the equivalent of dextran 10^4 Da, with most of it in a broad peak between about 10^5 and 10^6 Da.

Most of the added [^{14}C] XG oligosaccharide con-

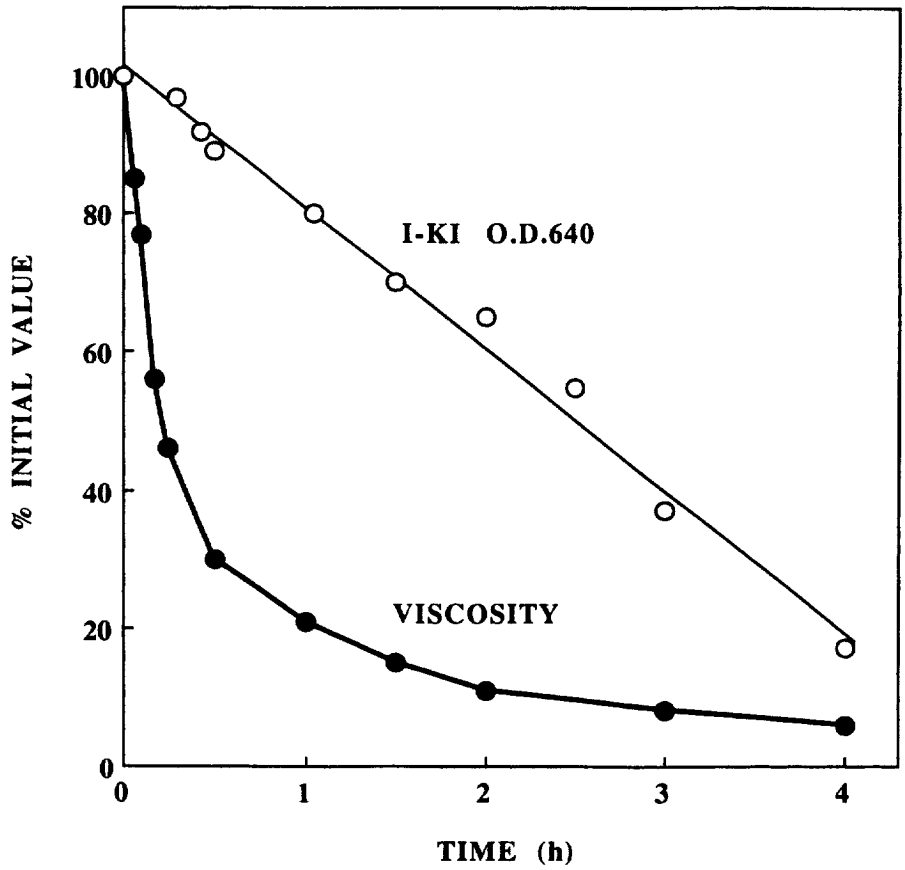

Fig. 4. Loss in viscosity (– ● –) and iodine color (– ○ –) by tamarind XG solution in the presence of purified *Trichoderma* cellulase. The cellulase (0.1 μg, from Megazyme) was added to 1.0 ml standard reaction mixture, pH 5.

Table 3. Reducing power of standard reaction mixtures during depolymerization of tamarind XG by *Trichoderma* cellulase (0.1 μg/ml) or green tomato extract (50 μg protein/ml)

Time	Reaction mixture	Reducing power* (μg Glc equiv/ml)		Viscosity (% initial)	
		Trichoderma	tomato	*Trichoderma*	tomato
Zero	complete	25	30	100	100
	-enzyme	24	25		
	-XG	26	28		
	-oligos	8	14		
1 hr	complete	30	30	16	20
2.5 hr	complete	42	31	7	14
5 hr	complete	64	25	5	13
4 days	complete	239	30	0	12
	-enzyme	24	25		
	-XG	25	28		
	-oligos	217	14		

*Values for reducing power are averages of 4 assays; average S.E. = ± 5% of values given.

tinued to elute near the V_t of the CL-6B column, but part (20%) became associated with the lower size range of depolymerized XG (Fig. 6), as expected from transglycosylation reactions of XET. The degree of incorporation of [^{14}C] from [^{14}C]oligosaccharides into XG fragments was more readily observed when the 3-day incubation mixture was passed through a column of Bio-Gel P_4. The profiles (Fig. 7) showed that almost

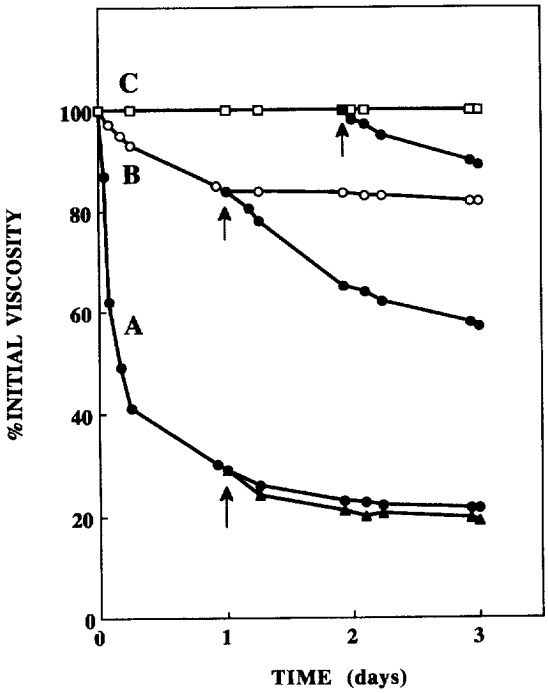

Fig. 5. Loss in viscosity of tamarind XG solutions. Fresh oligosaccharide (0.02%, final concentration) was added to reaction mixtures at the arrows. Curve A: Standard reaction mixture containing 1 μg salt-soluble green tomato protein, plus XG oligosaccharide at time zero (– ● –) and again at day 1 (-▲-). Curve B: As in curve A minus oligosaccharide from time zero (– ○ –). Curve C: Standard reaction mixture minus oligosaccharide containing only the high M_r fraction of tamarind XG that eluted from columns of Sepharose CL-6B in the void volume (– □ –) (see Refs. [4, 18, 19] and Fig. 6).

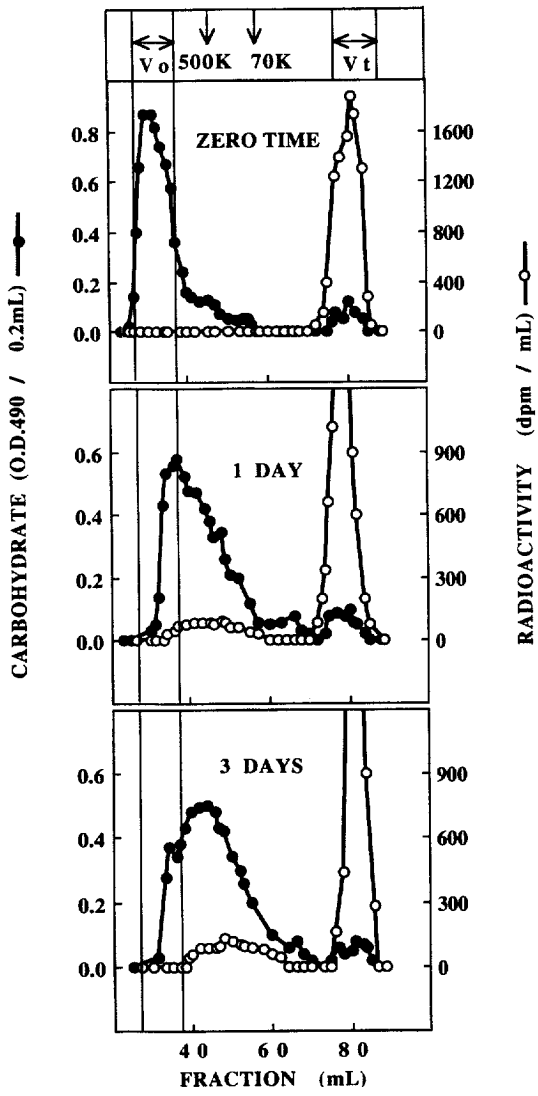

Fig. 6. Profiles of carbohydrate (– ● –) and radioactivity (– ○ –) following column chromatography through Sepharose CL-6B of reaction mixtures containing tomato enzyme, tamarind XG and [^{14}C]Fuc-labeled tamarind oligosaccharide. Viscosity losses in this test are shown in Fig. 3(B). Oligosaccharides were used at a specific activity of 50 dpm/μg. Columns were calibrated with dextrans ($V_o > 10^6$ Da) and Glc (V_t) and eluted with 0.1 M NaOH.

all of the carbohydrate was too large to be fractionated and emerged in the void of this column, along with 20% of the [^{14}C] that had transglycosylated. The labeled XG fragments that were formed in this experiment were about one tenth the initial size of TXG (Fig. 6). It can be calculated from the relative molar concentrations and the initial Mr of XG and XG oligosaccharides, that an incorporation of 20% (w/w) of oligosaccharides into XG by XET action should reduce the size of the XG donor to about the size observed.

These results (Figures 6–7) confirm that most XG oligosaccharide remained intact in the reaction mixture for at least 3 days and could not have been the factor limiting the ability of tomato enzyme to completely depolymerize tamarind XG. The enzyme was still potentially active even when viscosity did not decline further [Fig. 3(B) and Fig. 5, cf. Figure 6). The addition of a second dose of oligosaccharide, after the viscosity loss had stabilized, failed to evoke any further losses (Figure 5, curve A). It is concluded that the part of the tamarind XG that remained undegraded after 3 days incubation must have been resistant to depolymerization by the tomato enzyme. This is not surprising since even *Trichoderma* cellulase has difficulty hydrolysing highly galactosylated tamarind XG, as indicated by the fact that the trimer XXLG.XXLG.XLLG accumulates from tamarind XG in digestion media as a transient product before it eventually succumbs to hydrolysis [18]. Enzymes that degrade a XG chain at an unsubstituted Glc may be inhibited by adjacent isoprimeverose units that are galactosylated, as observed [23], for example, with some *Trichoderma* cellulases. The tomato enzyme would not have this problem with XG in tomato fruit which contains an unusually high proportion of unsubstituted Glc and very little Gal [24].

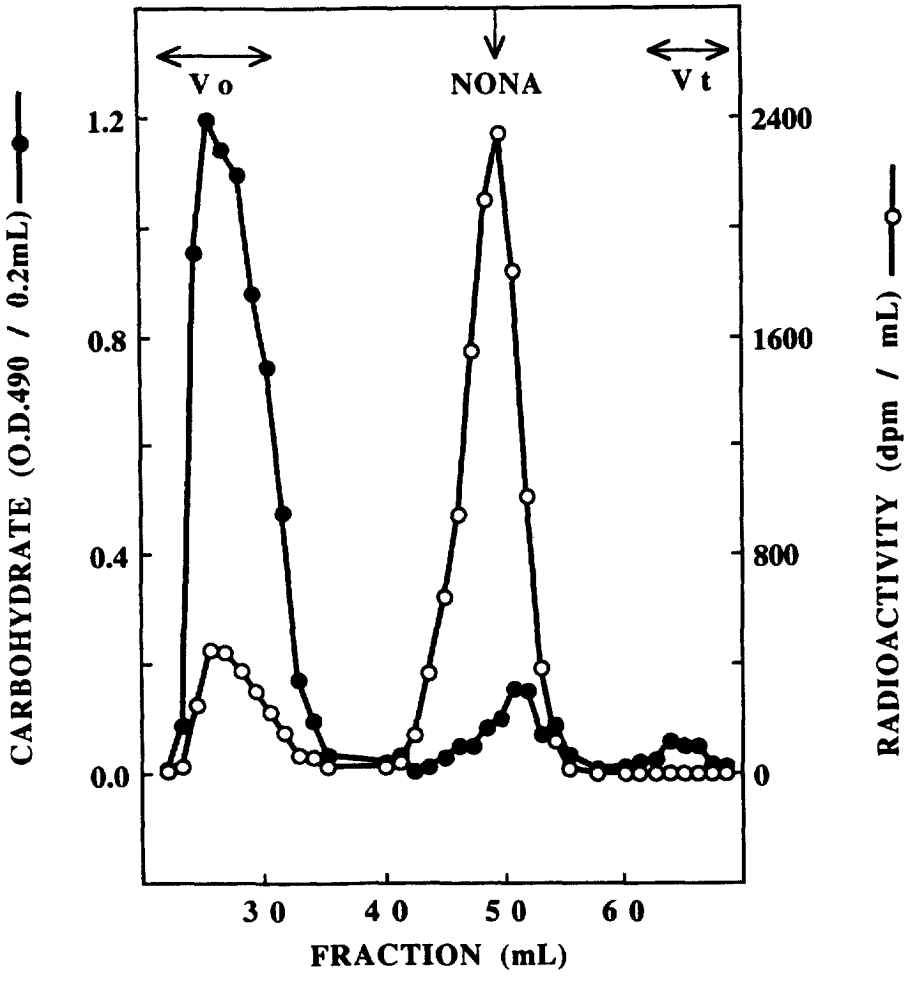

Fig. 7. Profiles following column chromatography through Bio-Gel P4 of reaction mixtures at 3 days as in Fig. 3(B) and Fig. 6, bottom panel. V_o represents fractions containing XG with $M_r > 10^4$ Da; V_t is the elution peak of Glc and the arrow labeled "nona" is the elution region of [^{14}C]XG oligosaccharides.

Reducing power of reaction mixtures

Standard reaction mixtures contained substantial reducing power due mainly to the oligosaccharides (0.02%) that were included Tab. 3. This reducing power increased markedly after *Trichoderma* cellulase was added but not after adding tomato extract in amounts that catalysed the same initial rate of viscosity loss of the tamarind XG solution. By one hour, the cellulase had reduced viscosity to 16% of initial levels and by four days there was no detectable viscosity above water values. Increased reducing power was detectable within an hour and reached a value by 4 days equivalent to about one-third of the potential oligosaccharide subunits in the XG of standard reaction mixtures. The tomato enzyme, in contrast, evoked no detectable increase in reducing power in 4 days, although viscosity had been reduced to an equilibrium of 12% of initial values. If this depolymerization had been due to hydrolysis, on extrapolation from the *Trichoderma* system, the reducing power should have increased by about one-third over initial values.

Conclusions

In every method used for separating protein in this study, the radiometric assay values for XET activity co-fractionated with the capacity for XG depolymerization as measured by viscosity loss (Figs. 1–2). Both assays indicated that the enzymic activity which they measured in young green tomato fruit was not extracted by buffer or detergent but required salt (e.g. 1 M NaCl) for solubilization (Table 1). These results do not prove but are consistent with the conclusion that the viscometric assay for XG degradation by

tomato extracts plus XG oligosaccharide was a measure of XET activity alone.

Progress curves for XG depolymerization by tomato salt extracts, whether assayed by loss of viscosity or iodine color (Fig. 3), or size distribution (Fig. 6), invariably stop short of complete degradation to oligosaccharide subunits which characterizes the reaction kinetics of cellulase action vs XG (Fig. 4). This is to be expected from enzyme that catalyses transglycosylation (XET) but not hydrolysis. To be sure, present results (Fig. 5) agree with earlier observations [1, 2, 16] that salt extracts of tomato fruit show a weak capacity to reduce the viscosity of tamarind XG solutions in the absence of added XG oligosaccharide. However, this was completely eliminated by using a preparation of tamarind XG with only very high M_r ($> 10^6$ Da) and removing all lower size ranges of XG fragments that could act as acceptor molecules for transglycosylation. This testifies against the presence in these tomato salt extracts of any significant β-glucanase activity and it confirms the conclusion of Nishitani [11] and de Silva *et al.* [16] that tomato XET does not act as a XGase in the absence of XG oligosaccharides. Finally, we could detect no increase in reducing chain ends of tamarind XG after incubation with tomato enzyme plus XG oligosaccharide for up to 4 days, by which time viscosity had been reduced by 88% (Table. 3). This is the result expected after cleavage by XET but not after hydrolysis.

We conclude that oligosaccharide-activated XGase activity is not detectable in salt-soluble extracts of young green tomato fruit, and XET activity is solely responsible for the depolymerization of XG that such extracts can evoke.

In vivo, during growth high M_r XG is intercalated into the expanding primary wall framework, and XET may function to promote incorporation of newly formed XG and to consolidate growth increments by transglycosylation of XG fragments to dislocated chains of bound XG. However, preparations of native wall XG always contain some chains with a relatively low M_r, including XG from green tomato fruit [2] and cell wall extracts of elongating tissue [25]. Free XG nonasaccharide has been detected in spinach cell cultures where it can accumulate to near micromolar concentrations [26]. Such fragments cannot be attributed to XET, but they could result from the action of 1,4-β-glucanases *in situ* (Fig. 2) or from incomplete chain lengthening during XG biosynthesis [27, 28]. If XET used such fragments as acceptors to catalyse XG depolymerization *in vivo*, the phenomenon might be localized and transient but useful, nonetheless, for evoking wall loosening.

EXPERIMENTAL

Materials and extracts

Purified XG from *Tamarindus indica* seed (TXG) and cellulase from *Trichoderma* sp. were purchased from Megazyme International Ireland. Oligosaccharide subunits from TXG were prepared by hydrolysis with *Trichoderma* cellulase [18] and fractionation on columns of Bio-Gel P2 (Bio-Rad). Using the nomenclature suggested by Fry *et al.* [29], the TXG used in this study contained four major oligosaccharide subunits XLLG, XLXG, XXLG and XXXG in a ratio by weight of 50:5:32:13 [2]. TXG was fucosylated using a solubilized pea XG fucosyltransferase [19] and the substrate GDP-L-[U-^{14}C] Fuc (8.3 GBq mmol^{-1}, New England Nuclear-Dupont). It was then hydrolysed with cellulase to produce an oligosaccharide mixture with the same subunits [^{14}C]fucosylated. The specific activity was 50 dpm/μg total oligosaccharide. Carboxymethylcellulose (CMC, type 7LP and 7HSP) was from the Hercules Powder Co. CM-TrisAcryl M was from Sepracor S.A., France and Con A-Sepharose from Pharmacia, Montreal. Most other specialized chemicals were purchased from Sigma. Cell wall ghosts were prepared from third internodes of week-old pea epicotyls by extracting with buffer and 4% NaOH–0.1% $NaBH_4$, which leaves a scoured XG:cellulose complex that retains the cell wall shape [21].

Tomatoes were grown in a greenhouse and, at the appropriate stage of development, fruits were detached, weighed, washed in 1% Na hypochlorite and frozen/stored at $-70°$. Enzyme extracts were prepared from thawed tomato fruit in the following sequence. Fruit was homogenized in an omnimixer (1 min, top speed) in one volume of cold extraction medium containing 20 mM NaPhosphate, pH 6.0, 5% glycerol, 1 mM DTT, 0.02% NaN_3 and the homogenate was immediately adjusted to pH 6.0 with NH_4OH. After stirring in the cold for 1 h, the suspension was centrifuged ($10{,}000 \times g$, 15 min) and the supernatant decanted through nylon mesh to produce a *buffer-soluble* enzyme extract. The insoluble residues were combined and rehomogenized in one volume of extraction medium containing 1% triton X-100. A few drops of 2-octanol were added to prevent foaming. The suspension was stirred and centrifuged to produce a *detergent-soluble* extract. The combined residues were then homogenized in one volume of extraction medium containing 1 M NaCl and a final *salt-soluble* extract prepared.

Enzyme assays

Depolymerization of β-glucan chains was assayed viscometrically [17] in Cannon-Manning semimicro viscometers in a final volume of 1.0 ml containing 40 mM NaPhosphate buffer, 0.01% NaN_3, 0.2 M NaCl and either 0.5% (w/v, final concentration) TXG or CMC. The pH was 6.0 when TXG was the substrate and either 5.0 or 7.0 when CMCase activity was assayed. The ratio of values for viscosity of the initial XG and CMC solutions which could be attributed to these polymers vs viscosity of the buffer medium alone in which they were dissolved (specific viscosity, η_{sp}),

ranged from 8 to 12. Tamarind subunit oligosaccharides were added at 0.02% (w/v final concentration), either before reaction was initiated with tomato extract or during the course of reaction. The % viscosity loss in a given time was calculated from the reduction observed in efflux time in the viscometer of the enzymic reaction mixture × 100 divided by the efflux time of the enzymic mixture minus the efflux time of the reaction medium. One unit of viscometric activity was defined here as the amount of enzyme that evoked 1% viscosity loss in 1 hr during the initial linear stage of reaction at 32° and optimum pH.

XET activity was assayed radiometrically [2, 4] in a reaction mixture (0.25 ml) containing the same ingredients and concentrations that were used in the viscometric assay, but with TXG oligosaccharide subunits, labeled with [^{14}C]Fuc. Activity was indicated by incorporation of [^{14}C] into material insoluble in cold 67% ethanol as a result of transglycosylation of cleaved XG fragments to [^{14}C]oligosaccharide acceptors. It has been shown [4, 6, 9, 30] that the common XG oligosaccharide subunits, including XXFG, all act as acceptors in the XET assay, albeit at different rates. One radiometric unit of XET activity was defined here as the amount of enzyme that conjugated 1% of the supplied [^{14}C]oligosaccharide with 67% ethanol-insoluble fragments of TXG in 1 hr during the initial linear stage of reaction at 32° and optimum pH (6.0).

The colorimetric assay that is specific for XG [20] involves the formation of a complex with iodine which develops a blue–green color (O.D. 640) when incubated in 20% Na_2SO_4. The color is most intense with long XG chains and declines as depolymerization proceeds [22]. The initial rate of loss of color, like the rate of viscosity loss, can measure the capacity for reducing XG chain length by either hydrolysis or transglycosylation.

When XG depolymerizes as a result of hydrolysis, the rate of reaction can be measured by the net rate of appearance of new reducing chain ends. Reducing power in there action media containing XG could not be assayed accurately by reduction of copper solution, e.g. the Fehlings test, because of long chains of XG precipitate in $Cu(OH)_2$. In present tests, reducing power was measured colorimetrically with alkaline *p*-hydroxybenzoic acid hydrazide [31]. One technical difficulty is the fact that the reducing power per mole of chains as long as those in TXG ($> 10^6$ Da) is very low compared to the reducing power per mole of TXG oligosaccharide subunits. In the typical reaction mixture used in present tests, hydrolysis of XG had to be extensive to increase the reducing power by a significant and detectable amount.

Depolymerization was observed directly by measuring the size distribution of carbohydrate in fractions produced by gel filtration through columns (100 × 1 cm) of Sepharose CL-6B or Bio-Gel P_4 calibrated with dextrans or oligosaccharides of known size. Sepharose columns were irrigated and eluted with degassed 0.1 M NaOH and Bio-Gel with 0.01% NaN_3. Carbohydrate was estimated with the phenol sulfuric acid reagent [32].

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