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ENZYMATIC DEGRADATION OF PECTIC SUBSTANCES AND CELL WALLS PURIFIED FROM CARROT CELL CULTURES

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Key Word Index—*Daucus carota*; Umbelliferae; cell wall; pectic substances; *exo*-polygalacturonase; *endo*-pectate lyase.

Abstract—The purified pectic substances from carrot (*Daucus carota*) cell cultures were depolymerized by purified *exo*-polygalacturonase and *endo*-pectate lyase. The degraded pectic fractions were fractionated by gel filtration chromatography, and the degree of polymerization and glycosyl composition determined for each fraction. The results indicate that subfractionated pectic substances contain a homogalacturonan region with a degree of polymerization of *ca* 70 and neutral glycosyl residues such as side-chains arranged in blocks ('hairy' regions) of different M_rs. In addition, the *endo*-pectate lyase released four pectic fragments from isolated cell walls. Based on the analysis of glycosyl composition of each fragment, the pectic substances of carrot cell walls are characterized.

INTRODUCTION

Pectic substances are found universally in the primary cell walls and intercellular layer of land plants. To date, the structural features of pectic substances have been studied by chemical analysis of fragments released from cell walls by partial acid hydrolysis and/or enzymatic degradation. Consequently, the prevailing concept is that pectic substances are constructed from a galacturonan, a rhamnogalacturonan, neutral polymers such as an arabinan, a galactan and an arabinogalactan, and several short side-chains [1]. The galacturonan and rhamnogalacturonan, which consist mainly of α-1,4-linked galacturonosyl residues, are attacked by endogenous pectolytic enzymes and the 'wall-modifying' enzymes secreted by phytopathogenic microorganisms [2]. In the ripening process, pectic substances of cell walls are converted *in situ* into soluble forms by the action of endogenous *endo*-polygalacturonase (acting in an *endo* fashion), resulting in extensive cell wall breakdown [3]. However, the polygalacturonase from root tissues and cell cultures of carrot [4], as well as that from apple cortical tissues [5], exhibit only *exo*-hydrolase (acting in an *exo* fashion) activity. The purified *exo*-polygalacturonase from carrot cell cultures do not degrade the corresponding cell wall preparation during *in vitro* incubation, and only partially degrade pectic substances extracted from the walls [6]. It is accepted that the action of *exo*-polygalacturonase is blocked by the presence of a neutral glycosyl moiety in the galacturonan chain [2]. In the light of these considerations, it is important to elucidate how neutral polymers and side-chains are distributed at the interior of the pectic backbone in carrot cell walls. The pectic substance structure of apple has been studied extensively in some detail [7–13], but that of carrot has received very little attention [14, 15].

In a preceding paper [16], we showed that the purified *endo*-pectate lyase from *Erwinia aroideae* was useful for the study of the gross structure of pectic substances within cell walls. Here, we have examined the enzymatic degradation of pectic substances and cell walls purified from carrot cell cultures; the enzymes used were *exo*-polygalacturonase (poly-1,4-α-D-galacturonide glycanohydrolase, EC 3.2.1.67) and *endo*-pectate lyase (poly-1,4-α-D-galacturonide lyase, EC 4.2.2.2).

RESULTS AND DISCUSSION

Characteristics of cell walls and pectic substances

The cell walls used in this experiment were isolated from 15-day-old cell cultures corresponding to the end of the exponential phase of growth cycle [6]. The cell wall materials (crude cell walls) obtained from sonic-treated cell homogenates were prepared by successive washing with hot ethanol, acetone and ether [6]. Finally, the crude cell walls were treated with protease and α-amylase to remove protein and starch [16]. Five grams of lyophilized cell cultures yielded 1.2 g (dry wt) of crude cell walls and 0.9 g (dry wt) of starch-free cell wall preparation. Pectic substances were extracted from the starch-free cell wall preparation with hexametaphosphate. The glycosyl compositions of cell walls and pectic substances are shown in Table 1. The noncellulosic neutral glycosyl and uronosyl residues of cell walls were 30.0% and 6.3%, respectively. All the uronosyl residues in the cell walls appear to be galacturonosyl residues; glucuronic acid was not detected by PC, although the sensitivity of the method is not high. As reported previously [6], the extracted pectic substances consisted of galacturonosyl and neutral glycosyl residues, predominantly rhamnosyl, arabinosyl and galactosyl res-

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Table 1. Glycosyl compositions of cell walls and pectic substances from cell cultures of carrot

Glycosyl residue*	Cell walls (mg/100 mg dry wt of cell walls)	Pectic substances (wt % total sugars)
Rhamnose	3.1	10.6
Fucose	0.7	0.2
Arabinose	11.7	47.0
Xylose	3.0	3.3
Mannose	2.8	0.5
Galactose	5.8	13.0
Glucose	3.2	2.6
Galacturonic acid	6.3	22.8

*Data represent the mean of three analyses, each run in duplicate.

idues, suggesting a composition typical for pectic substances and associated sugars [1].

Degradation of fractionated pectic substances by exo-*polygalacturonase*

The extracted pectic substances were separated into four fractions by DEAE-Sephadex A-50 ion exchange column chromatography, the major fraction (P-3) of which was further fractionated into two carbohydrate fractions, P-3A and P-3B, by Bio-Gel A-5m gel filtration chromatography. From the glycosyl compositions of each fraction described in a previous paper [6], carrot pectic substances can be classified into three types; the first type is a neutral polymer such as arabinan or arabinogalactan containing a high proportion of arabinosyl residues (*ca* 89 wt %); the second type is a rhamnogalacturonan covalently binding many long and/or short side-chains which comprise a high arabinosyl-to-galactosyl residues ratio (*ca* 50 wt %); the third type is a galacturonan core containing a high proportion of galacturonosyl residues (more than 80 wt %). It may be concluded that pectic substances extracted from carrot cell walls contain the three types in a ratio of 1:4:3. Perhaps, because these polymers can be easily extracted with hexametaphosphate from the cell walls and fractionated by a combination of chromatographic techniques including ion exchange and gel filtration, they bind less tightly in each other. Parts of the second type (fraction P-3A) and third type (fraction P-3B) were further characterized.

Fraction P-3A is rich in neutral glycosyl residues (69.5 wt %), whereas fraction P-3B is rich in galacturonosyl residue (79.6 wt %). The apparent M_rs of fractions P-3A and P-3B, as determined by gel filtration on a Bio-Gel column by comparison with the elution volume of linear dextrans (Pharmacia) of known M_r, are greater than 5×10^5 and *ca* 5×10^4, respectively. When fractions P-3A and P-3B were reacted with the purified *exo*-polygalacturonase, the reaction products were only galacturonic acid as revealed by PC. The degradation, which calculated from the increase in reducing power released by the enzyme action, resulted in hydrolysis of *ca* 8% and 29% of the glycosyl linkages of fractions P-3A and P-3B, respectively. To determine the change in M_r, the enzyme-treated fractions P-3A and P-3B were dialysed against water in order to remove the galacturonic acid as reaction products and subjected to gel filtration on a Bio-Gel A-5m column, and then the carbohydrate in each fraction was eluted as a single peak. The elution profile of fraction P-3A was almost the same as that of native fraction P-3A. While fraction P-3B showed a slight reduction in M_r after the enzyme treatment, indicating that it had an M_r of *ca* 3.6 $\times 10^4$. In any case, the molecular sizes were not significantly reduced by the action of the enzyme. It is well known that *exo*-polygalacturonase catalyses the hydrolytic cleavage of the terminal α-1,4-linkages of galacturonosyl polymers, releasing galacturonosyl units [2]. Therefore, these fractions, in particular fraction P-3A, would be a structurally heterogeneous complex polymer consisting of many kinds of galacturonan backbone [17]. Albersheim and co-workers [18, 19] have reported that a homogalacturonan region is present in sycamore cell walls from the analyses of fragments obtained by *endo*-polygalacturonase treatment and partial acid hydrolysis, but the length of this polymer ('smooth' region) is still obscure. The data of Chambat and Joseleau [20] also demonstrate that a homogalacturonan with a degree of polymerization greater than 100 has been obtained from the cell walls of *Rosa* cell cultures with base treatment. If we assume linear structural feature for the polymer in fraction P-3B, it appears that the polymer contains a homogalacturonan region with a degree of polymerization of *ca* 70 as deduced by gel filtration chromatography after *exo*-polygalacturonase treatment.

Degradation of exo-*polygalacturonase-treated fractions P-3A and P-3B by* endo-*pectate lyase*

The *exo*-polygalacturonase-treated polymers of fractions P-3A and P-3B, referred to hereafter as P-3A-PG and P-3B-PG, were submitted to the action of *endo*-pectate lyase. After heat inactivation of the enzyme, the reaction mixtures were subjected to gel filtration on a Bio-Gel A-5m column. The enzyme released *ca* 7% and 37% of carbohydrate fraction as reaction products from fractions P-3A-PG and P-3B-PG, respectively (Figs 1A and

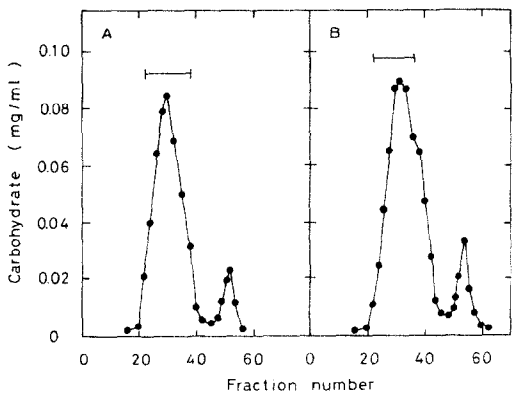

Fig. 1. Gel filtration chromatography of fraction P-3A-PG on Bio-Gel A-5m after *endo*-pectate lyase treatment. The sample was treated with the purified enzyme, placed on the column (1.8 × 90 cm) and filtered with 50 mM acetate buffer (pH 5.2) containing 20 mM EDTA at a flow rate of 0.2 ml/min. The eluate was collected in 3 ml fractions and the fractions were assayed for total sugars by the phenol–H_2SO_4 method. A, Native fraction P-3A-PG (3.5 mg sugar content); B, de-esterified fraction P-3A-PG (4.3 mg sugar content).

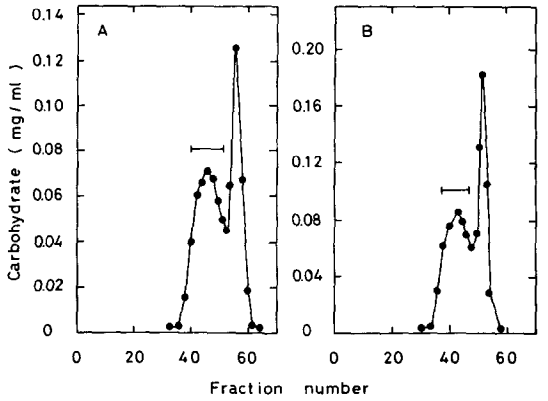

Fig. 2. Gel filtration chromatography of fraction P-3B-PG on Bio-Gel A-5m after *endo*-pectate lyase treatment. The sample was treated with the purified enzyme and chromatographed by the same procedure as in Fig. 1. A, Native fraction P-3B-PG (3.8 mg sugar content); B, de-esterified fraction P-3B-PG (5.1 mg sugar content).

2A). The fraction P-3B-PG showed a slight reduction in M_r after the enzyme treatment, indicating that it had an M_r of *ca* 2.5×10^4. It is possible that the methylesterified galacturonosyl residues in these fractions block further degradation by the enzyme. Thus, the fractions P-3A-PG and P-3B-PG were de-esterified by treatment with base [21] prior to enzyme treatment. The reaction product released from de-esterified fraction P-3A-PG increased by only 3 % over that of untreated fraction P-3A-PG, and the elution profile on Bio-Gel A-5m was changed significantly by the enzyme action (Fig. 1B). Whereas the degradation of de-esterified fraction P-3B-PG was the same as with untreated fraction P-3B-PG (Fig. 2B). This implies that the methoxyl groups are distributed along the molecule in fraction P-3A-PG.

The apparent M_r of the reaction products released from fractions P-3A-PG and P-3B-PG was below 1×10^4. When the reaction products were hydrolysed completely with trifluoroacetic acid, the hydrolysates were predominantly galacturonosyl residues. On the other hand, the percentages of galacturonosyl residues in fractions P-3A-PG and P-3B-PG acted upon by the enzyme, in particular fraction P-3B-PG, were clearly reduced, but those of neutral glycosyl residues except for xylosyl and glucosyl residues were slightly increased relatively (Tables 2 and 3). Barrett and Northcote [7] have reported that apple pectic substances can be cleaved into two components by a transelimination reaction with hot sodium phosphate buffer (pH 6.8). Furthermore, they demonstrated the characteristics of the two components fractionated by Sephadex gel filtration. Our data, obtained from the *endo*-pectate lyase treatment, are very similar to those from the chemical degradation as described above. It is particularly noteworthy that the *endo*-pectate lyase released the region of galacturonosyl polymer binding xylosyl and glucosyl residues from fractions P-3A-PG and P-3B-PG.

The action pattern of the two enzymes towards carrot pectic substances suggests that most of the neutral glycosyl residues are concentrated in a 'hairy' region, which is not degraded by the pectolytic enzymes, and are not dispersed in pectic molecule. This is in agreement with the idea by De Vries *et al.* [11, 12] on apple pectic substances. In addition, it is interesting to note that the

Table 2. Glycosyl compositions of native and de-esterified fraction P-3A-PG after *endo*-pectate lyase treatment*

	Wt % of total sugars		
		Enzyme treated	
Glycosyl residue	P-3A-PG	Native	De-esterified
Rhamnose	13.3	14.9	15.4
Fucose	0.2	0.3	0.4
Arabinose	40.0	43.5	45.3
Xylose	5.3	4.0	2.8
Mannose	0.3	0.6	0.3
Galactose	10.2	12.4	16.1
Glucose	1.9	1.2	1.5
Galacturonic acid	28.8	23.3	18.2

*Data represent the mean of three analyses, each run in duplicate.

Table 3. Glycosyl compositions of native and de-esterified fraction P-3B-PG after *endo*-pectate lyase treatment*

	Wt % of total sugars		
		Enzyme treated	
Glycosyl residue	P-3B-PG	Native	De-esterified
Rhamnose	4.4	9.2	8.8
Fucose	0.3	0.8	0.8
Arabinose	14.5	22.9	19.2
Xylose	3.4	3.2	2.4
Mannose	0.3	0.3	0.5
Galactose	4.5	8.3	8.2
Glucose	1.7	1.6	1.3
Galacturonic acid	70.8	53.8	58.7

*Data represent the mean of three analyses, each run in duplicate.

'hairy' region could be grouped into two types of polymers with a different degree of polymerization. In our view, pectic substances in carrot cell cultures are of a more complicated structure as a whole than apple pectic substances.

Degradation of isolated cell walls by endo-*pectate lyase*

The *endo*-pectate lyase used in this experiment was able to attack the cell walls directly and solubilized effectively pectic fragments as the reaction products [16]. To understand the structural feature of pectic substances within cell walls, therefore, the cell walls from carrot cell cultures were submitted to the action of the *endo*-pectate lyase. After exhaustive enzyme treatment, the reaction products were 23.1 mg (sugar content) from 100 mg (dry wt) cell walls and galacturonosyl residue was not detected in the wall residues. This strongly suggests that the enzyme solubilizes completely the pectic fraction of the cell walls. When the pectic fragments released were subjected to gel filtration on a Bio-Gel P-2 (Bio-Rad) column (1.8×90 cm; $V_0 = 57$ ml, $V_i = 141$ ml), the carbohydrate was eluted near the void volume following slight

'tail' (data not shown). Thus, most of the pectic fragments solubilized had comparatively high M_rs. It may be deduced that neutral glycosyl residues covalently binding to galacturonan chains would hinder further degradation of pectic fragments by enzyme action.

The pectic fragments (114 mg sugar content) were then fractionated by DEAE-Sephadex A-50 ion exchange column chromatography yielding the four carbohydrate fractions (F-1, F-2, F-3 and F-4) illustrated in Fig. 3. The four fractions were pooled separately, and salt was removed from each fraction by Bio-Gel P-2 gel filtration. This procedure yielded 21.7 mg of fraction F-1, 16.2 mg of fraction F-2, 11.8 mg of fraction F-3 and 33.1 mg of fraction F-4. Thus, the eluted fractions accounted for *ca* 73% of the starting material. Fraction F-2 was a fragment containing more than 95% galacturonosyl residues as a homogalacturonan, which corresponded to 19.6% of the recovered materials and accounted for 4.5% of the original wall samples. Fractions F-1, F-3 and F-4 had different glycosyl compositions (Table 4). Fraction F-1 comprised a high neutral glycosyl composition such as arabinosyl residues, so that the fraction would come from a part of either an arabinan or arabinogalactan. An apparent content of galacturonosyl residues clearly demonstrates that the neutral polymers are covalently attached to galacturonan chains without a covalent connection to other cell wall constituents. Fraction F-3 comprised a high arabinosyl to galacturonosyl residues ratio similar to the glycosyl composition of fraction P-3A-PG. Fraction F-4 which represented 40.0% of the recovered materials appeared to be a major polymer of the pectic substances. It consisted of equal amounts of rhamnosyl and galacturonosyl residues, suggesting a composition typical for a rhamnogalacturonan.

In conclusion, our data suggest that the 'hairy' regions contribute to the structural stability of the pectic backbone. In other words, the 'hairy' regions could play an important role in controlling pectic substance degradation *in situ* by *exo*-polygalacturonase in carrot cells. For many years, the suggestion has been made [3, 7] that the pectic substances serving structural elements of cell walls undergo alternations in amount and/or structure during cell growth and differentiation. Therefore, we must further consider the question whether glycosidases other than pectolytic enzymes are involved in the degradation of the 'hairy' regions in pectic substances.

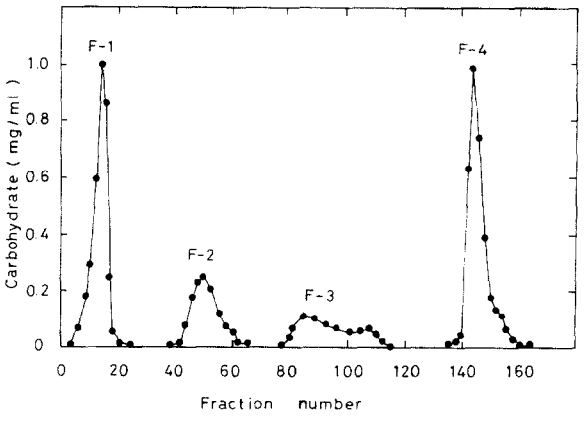

Fig. 3. DEAE-Sephadex A-50 ion exchange column chromatography of pectic fragments as reaction products released from carrot cell walls by *endo*-pectate lyase. Three ml of the reaction products (114 mg sugar content) was applied to a DEAE-Sephadex column (2.0 × 20 cm) which had been equilibrated with 25 mM phosphate buffer (pH 6.0). The column was eluted with 25 mM (fractions 1–30), 100 mM (fractions 31–70), 250 mM (fractions 71–130) and 500 mM (fractions 131–170) phosphate buffer, respectively. The eluate was collected in 5 ml fractions which were assayed for total sugars by the phenol–H_2SO_4 method.

Table 4. Degradation of isolated carrot cell walls by *endo*-pectate lyase*

	Wt % of total sugars		
Glycosyl residue	F-1	F-3	F-4
Rhamnose	2.1	14.1	17.7
Fucose	2.6	Tr†	Tr
Arabinose	38.7	41.5	46.9
Xylose	15.0	3.0	4.4
Mannose	3.1	ND†	ND
Galactose	13.2	10.5	10.5
Glucose	12.3	Tr	0.6
Galacturonic acid	13.0	30.8	19.9

*Data represent the mean of three analyses, each run in duplicate.

†Tr, Trace: ND, none detected.

EXPERIMENTAL

Preparation of cell walls and pectic substances. Cell suspension cultures of carrot (*Daucus carota* L. cv. Kintoki) were cultured in the medium of Murashige and Skoog [22] containing 3% (w/v) sucrose as the carbon source and 4.5 μM 2,4-dichlorophenoxyacetic acid as a growth regulator [4]. The cell walls were prepared from 15-day-old cultures, treated with Pronase (Kaken Chemical) and pancreatic α-amylase (Sigma Chemical). They were then stored in a desiccator over $CaCl_2$ until required. Pectic substances were extracted from the starch-free cell wall preparation as described previously [6], using hot NahexametaPi soln [23].

Purification of pectic substances. Pectic substances were fractionated by DEAE-Sephadex A-50 (Pharmacia) ion exchange chromatography as described previously [6]. The fractionated pectic polymer (fraction P-3) was dissolved in 50 mM NaOAc buffer (pH 5.2) containing 25 mM EDTA and subjected to gel filtration on a Bio-Gel A-5m (Bio-Rad) column (1.8 × 90 cm; V_0 = 66 ml, V_i = 189 ml), which had been equilibrated with the same buffer. The carbohydrate was filtered with the same buffer at a flow rate of 0.2 ml/min. Fractions of 3 ml were collected and carbohydrate estimated by the PhOH–H_2SO_4 method [24]. Two carbohydrate fractions (P-3A and P-3B) were pooled separately, dialysed against H_2O, concd with evaporation *in vacuo* at 40° and redissolved in 50 mM NaOAc buffer (pH 5.2) containing 25 mM EDTA. Each fraction was further purified by Bio-Gel A-5m gel filtration chromatography, conditions as just described, and the fractions containing carbohydrate pooled.

Sources and purification of enzymes. exo-Polygalacturonase was purified from homogenates of cell cultures of carrot by a procedure involving dialysis at pH 5.2, chromatography on DEAE-Sephadex A-50 and on Sephadex G-150 (Pharmacia) and

prep. PAGE, as described previously [6]. One unit of enzyme activity is defined as the amount that forms 1 μmol galacturonic acid from 0.2% (w/v) acid-insoluble polygalacturonate [4] per hr at pH 5.0 and 37°. *endo*-Pectate lyase was purified from cell homogenates of *Erwinia aroideae* by a procedure involving $(NH_4)_2SO_4$ fractionation, CM-Sephadex C-50 (Pharmacia) batch wise, and chromatography on CM-Sephadex C-50 and Sephadex G-200 (Pharmacia), as described previously [16]. *E. aroideae* was grown on a liquid medium (pH 7.2), which contained 0.5% (w/v) pectic acid, 0.5% (w/v) peptone, 0.3% (w/v) meat extract, 15 mM KPi and 28 mM NaPi, with constant shaking at 27° for 4 days. One unit of enzyme activity is defined as the amount that forms 1 μmol aldehyde group from 0.2% (w/v) acid-insoluble polygalacturonate soln containing 0.5 mM $CaCl_2$ per min at pH 8.6 and 37°.

Enzyme treatment of pectic substances and cell walls. Pectic polymers, fractions P-3A and P-3B (4–7 mg sugar content) were incubated at 30° with the purified *exo*-polygalacturonase (3.7 units) and 50 mM NaOAc buffer (pH 5.0). A drop of toluene was added to suppress microbial growth. After incubation for 30 hr, the reaction was stopped by heating in a boiling water bath for 3 min and the formation of reducing groups assayed by the Somogyi method [25]. *exo*-Polygalacturonase-treated fractions P-3A and P-3B were dialysed against H_2O and then incubated at 30° with the purified *endo*-pectate lyase (2.8 units), 0.5 mM $CaCl_2$ and 50 mM Tris–HCl buffer (pH 8.6) under toluene. After incubation for 30 hr, the reaction mixture was adjusted to pH 5.0 with 0.1 M HOAc and heated in a boiling water bath for 3 min. For *endo*-pectate lyase treatment of cell walls, the cell walls (10 mg dry wt) were incubated at 30° with the enzyme (0.9 unit), 0.5 mM $CaCl_2$ and 50 mM Tris–HCl buffer (pH 8.6) under toluene, with continuous stirring. After incubation for 30 hr, the reaction products were separated from the wall residues by filtration on glass fibre filter paper. The reaction products were adjusted to pH 5.0 and heated in a boiling water bath for 3 min.

Analyses. Neutral glycosyl residues of cell walls and pectic substances were analysed by GC as their alditol acetate derivatives according to the technique of ref. [26]. GC was performed with a Hitachi instrument equipped with a Chromato-Processor as described previously [27]. Galacturonic acid was estimated by the carbazole-H_2SO_4 method [28].

De-esterification of pectic substances. The sample (3–5 mg sugar content) was dissolved in 9 ml H_2O. 1 ml 0.1 M NaOH was added and, after gently stirring for 90 min in an ice-water bath, the soln was adjusted to pH 5.0 with 0.1 M HOAc and dialysed against H_2O [21].

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