Pergamon

PII: S0031-9422(96)00883-7

Phytochemistry, Vol. 45, No. 4, pp. 627–632, 1997

0031-9422/97 $17.00+0.00

SYNTHESIS OF CELL-WALL GLYCOPROTEINS AND THEIR CHARACTERIZATION IN OAT COLEOPTILES

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(*Received in revised form* 30 *October* 1996)

Key Word Index—*Avena sativa*; Gramineae; oat coleoptiles; biosynthesis; cell-wall glycoproteins; D-glucosamine metabolism; tunicamycin; deoxynojirimycin; deoxymannojirimycin.

Abstract—D-[U-^{14}C]Glucosamine was rapidly taken up by oat coleoptile segments and metabolized to radioactive UDP-*N*-acetylglucosamine, which acted as specific glycosyl donor for the synthesis of glycolipids and cytosolic, membrane-bound and cell-wall glycoproteins. Cell-wall glycoproteins were solubilized from the walls by either cell-wall-degrading enzymes or chemical extractants. The solubilized cell-wall glycoproteins in the presence of peptide *N*-glycosidase F released oligosaccharide chains higher than seven glycosidic residues. The combined action of peptide *N*-glycosidase F and *N*-acetyl-β-D-glucosaminidase on cell-wall glycoproteins indicated the presence of *N*-acetylglucosamine residues β-1,2-linked to mannose. Less than 9% of the radioactive oligosaccharide chains was released from the solubilized cell-wall glycoproteins when treated with 0.5 M NaOH at 20°, whereas more than 45% of the radioactivity was released in the presence of 1 M NaOH at 50°. The high hydrolytic sensitivity of cell-wall glycoproteins to peptide *N*-glycosidase F, *N*-acetyl-β-D-glucosaminidase and NaOH at 50° indicated that most *N*-acetylglucosamine residues were incorporated into *N*-linked cell-wall glycoproteins. Further evidence of this was obtained by the use of inhibitors of biosynthesis and processing of *N*-linked glycoproteins. ©1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

It has been demonstrated that in excised bean and corn root tips [1], seedlings of *Calluna* [2], barley and oat coleoptile segments [3] and carrot root disks [4], exogenously supplied D-[U-^{14}C]glucosamine is rapidly taken up by the cells and sequentially metabolised to radioactive *N*-acetylglucosamine, *N*-acetylglucosamine 6-phosphate, *N*-acetylglucosamine 1-phosphate, UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine. In addition, UDP-*N*-acetylglucosamine acts as a specific glycosyl donor for the synthesis of glycolipids and *N*- and *O*-linked glycoproteins [3, 5–8]. *N*- and *O*-linked glycoproteins together with structural polysaccharides, such as pectins, hemicelluloses and cellulose, are components of the plant cell-wall. This compartment changes constantly and coordinately according to the developmental stages of the cell and exposure to any abiotic and biotic stress. Some of these changes are controlled at the level of genes encoding cell-wall proteins [9–12].

We have previously reported that radioactive *N*-acetylglucosamine residues are incorporated into cytosolic, membrane and cell-wall glycoproteins, as well as glycolipids of oat coleoptile segments incubated in the presence of D-[U-^{14}C]glucosamine [3]. In the present work, we describe (a) the solubilization of the glycoproteins containing radioactive *N*-acetylglucosamine residues from purified cell-walls by the use of either polysaccharide- or protein-degrading enzymes or chemical extractants; (b) the partial characterization of the oligosaccharide chains of the synthesised cell-wall glycoproteins and (c) the effect of inhibitors on the biosynthesis and processing of *N*-linked cell-wall glycoproteins.

RESULTS AND DISCUSSION

Preliminary experiments have shown that exogenously supplied D-[U-^{14}C]glucosamine is rapidly taken up by the cells of oat coleoptile segments and metabolized to radioactive UDP-*N*-acetylglucosamine, which is epimerized to small amounts of UDP-*N*-acetylgalactosamine. UDP-*N*-acetylglucosamine acts as a specific glycosyl donor for the synthesis of oligosaccharide chains of membrane glycolipids and cytosolic, membrane and cell-wall glycoproteins [3]. Therefore, we have used this experimental system in order to study the synthesis and characterization of cell-wall glycoproteins.

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Table 1. Solubilization of cell-wall glycoproteins using polygalacturonase, driselase, cellulase and protease. Purified cell-walls were isolated from oat coleoptile segments which had been incubated under sterile condition in water containing 111 kBq of D-[U-^{14}C]glucosamine for 4 hr at 20° in the dark. The results are from one representative experiment of three

Time (hr)	Radioactivity (%)			
	Polygalacturonase	Driselase	Cellulase	Protease
2	27.3	41.4	41.5	42.3
4	nd*	27.9	18.2	25.0
6	4.5	8.6	14.0	17.4
8	5.3	4.0	10.3	5.7
10	5.3	1.4	6.9	1.2
24	0.9	0.8	2.2	0.3
Insoluble residue	56.7	15.9	6.9	8.1
Total radioactivity (Bq)	1081	564	554	845

* nd, not determined.

Table 1 shows the solubilization of glycoproteins containing radioactive *N*-acetylglucosamine residues from purified cell-walls by the use of cell-wall-degrading enzymes, such as polygalacturonase, Driselase (a mixture of cell-wall-degrading enzymes used for the preparation of plant protoplasts), cellulase and protease. Driselase, cellulase and protease solubilized from the purified cell-walls 84–93% of the total ^{14}C within 10 hr of incubation. By using polygalacturonase, *ca* 43% of radioactive glycoproteins was solubilized. The four types of cell-wall-degrading enzymes solubilized only small amounts of radioactivity after 10 and 24 hr of incubation. The solubilized cell-wall glycoproteins were immobile on high voltage paper electrophoresis (pH 2.0, 5 kV, 45 min) and PC (solvent system: A). Although the enzymic digestion of pectins increases cell-wall porosity, the capability of polygalacturonase to solubilize cell-wall glycoproteins was lower than that of Driselase, cellulase and protease. Our results could be explained from the notion, that in monocotyledons, the amount of pectins in the wall is much less represented in comparison with hemicelluloses and cellulose microfibrils [13]. Consequently, it would be more difficult to hydrolyse the walls using only polygalacturonase for the release of the wall glycoproteins. In addition, it has to be taken into consideration that the polygalacturonans, which are mainly located in the middle lamella, may be less accessible than other polysaccharides to the hydrolytic action of the exogenously-supplied polygalacturonase.

The solubilized cell-wall glycoproteins obtained with either cellulase or protease were treated with peptide *N*-glycosidase F. This glycosidase recognizes the *N*-glycosidic bonds of high mannose, complex and hybrid types with high specificity. The reaction mixture was passed through Centricon 10 to separate oligosaccharide from polypeptide chains; *ca* 90% of the radioactivity was recovered in the filtrate. The same results were obtained when the reaction mixture was treated with 80% cold ethanol to precipitate the protein fraction; the *N*-glycan supernatant was collected and scanned for radioactivity. This evidence proved that most of the oligosaccharide chains of the cell-wall glycoproteins is *N*-linked. The radioactive *N*-glycans moved on PC (solvent system: A) slightly slower than the maltoheptaose marker. This indicates that the *N*-glycans are oligosaccharide chains having more than about seven glycosyl residues. The combined action of peptide *N*-glycosidase F and *N*-acetyl-β-D-glucosaminidase (the last one at low enzyme concentration) on the cellulase- and protease-solubilized cell-wall glycoproteins hydrolysed radioactive *N*-glycans, which moved on PC (solvent system: A) as maltotri-, maltotetra- and maltoheptaoligsaccharide markers (Fig. 1). These results suggested the presence of *N*-acetylglucosamine, β-1,2-linked to mannose in the radiolabelled *N*-glycans. These linkages are specifically hydrolysed by *N*-acetyl-β-D-glucosaminidase, when the enzyme is used at low concentration [14]. Our data indicate that oat cell-wall glycoproteins have a complex type of oligosaccharide chains. Similar characteristics have been demonstrated in the oligo-

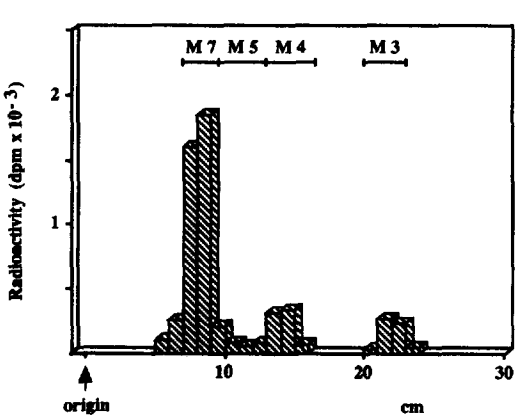

Fig. 1. Pattern of radioactive oligosaccharides released by peptide *N*-glycosidase F and *N*-acetyl-β-D-glucosaminidase on cell-wall glycoproteins. M 7, maltoheptaose; M 5, maltopentaose; M 4, maltotetraose; M 3, maltotriose.

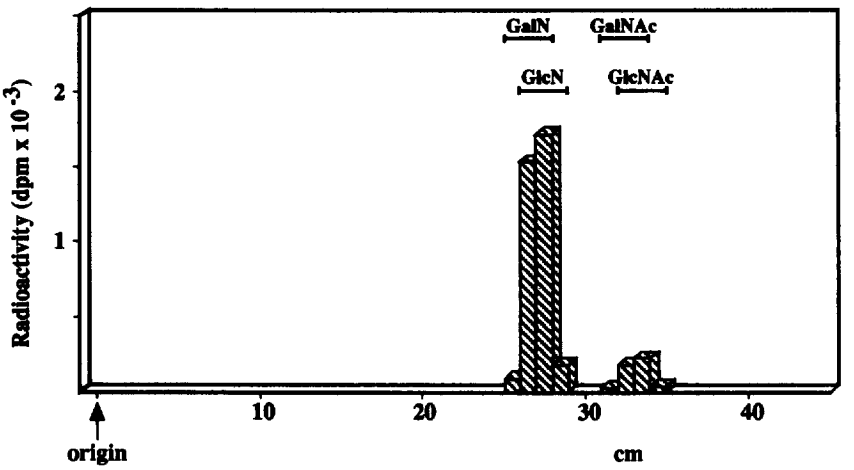

Fig. 2. PC separation of radioactive *N*-glycans hydrolysed with 3 M HCl. GlcN, glucosamine; GalN, galactosamine; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

saccharide chains of laccase, a glycoprotein isolated from the medium of suspension-cultured sycamore cells (*Acer pseudoplatanus*); these oligosaccharide chains have terminal fucose and galactose residues β-1,4- and β-1,6-linked to the outermost *N*-acetylglucosamine residue, which is β-1,2-linked to mannose [15].

The radioactive *N*-glycans hydrolysed with 3 M HCl gave only D-[U-^{14}C]glucosamine (*ca* 90%) and *N*-acetyl-D-[U-^{14}C]glucosamine (*ca* 10%) residues as determined by PC (solvent system: B) (Fig. 2). No radioactive galactosamine was detected when the radioactivity corresponding with glucosamine and galactosamine markers was eluted and rechromatographed (PC solvent system: C) for the separation of the two amino sugars. The ratio between D-[U-^{14}C]glucosamine: *N*-acetyl-D-[U-^{14}C]glucosamine decreased when milder conditions of acid hydrolysis were used.

The cell-wall glycoproteins containing radioactive residues of *N*-acetylglucosamine were rapidly and efficiently solubilized by either SDS or a mixture of phenol–acetic acid–water (2:1:1, w/v/v). One per cent SDS at 95° extracted *ca* 95% of cell-wall radioactive glycoproteins after 15 min without degradation of proteins as judged by SDS-PAGE and autoradiography. By treatment with phenol–acetic acid–water (2:1:1, w/v/v), performed at 70° for 30 min, *ca* 90% of the radioactive cell-wall glycoproteins were extracted, without degradation of proteins, as monitored by SDS-PAGE and autoradiography. The SDS-solubilized cell-wall glycoproteins were characterized by treatment with NaOH–$NaBH_4$ at two different concentrations and temperatures. In the presence of 0.5 M NaOH–1 M $NaBH_4$ at 20°, the hydrolysed radioactive *N*-glycans were less than 8%. A stronger concentration of NaOH (1 M) and a higher temperature (50°) were required in order to hydrolyse *ca* 45% of the radioactive *N*-glycans. The results confirm that the radioactive *N*-acetylglucosamine residues were mainly incorporated into the oligosaccharide chains of *N*-linked glycoproteins; similar results have been obtained using rice coleoptile cell-walls [16]. However, our data do not exclude the possibility that some *N*-acetylglucosamine residues may be *O*-linked in cell-wall polymers.

Another approach to confirm the incorporation of radioactive *N*-acetylglucosamine residues into *N*-linked cell-wall glycoproteins and the presence of different *N*-glycans in the glycoproteins secreted into the cell-wall, was that of studying the biosynthesis and processing of *N*-linked oligosaccharide chains in the presence of inhibitors.

The uptake of D-[U-^{14}C]glucosamine by oat coleoptile segments after 2 hr of incubation was *ca* 21% of the initial radioactivity (32 kBq). When segments were incubated with tunicamycin for 3 hr and D-[U-^{14}C]glucosamine was added during the last 2 hr of incubation, the uptake of D-[U-^{14}C]glucosamine was stimulated by 17% using 10 μM tunicamycin.

D-[U-^{14}C]Glucosamine taken up by the cells was sequentially metabolised to *N*-acetylglucosamine, *N*-acetylglucosamine 6-phosphate, *N*-acetylglucosamine 1-phosphate and UDP-*N*-acetylglucosamine (Table 2). Metabolism of the amino sugars was little affected quantitatively by tunicamycin. Significantly, the percentage of radioactive UDP-*N*-acetylglucosamine increased in the presence of tunicamycin at all concentrations tested. All the incorporation data were calculated as per cent with respect to the uptake of radioactive D-glucosamine in the different treatments. The inhibitory effect of tunicamycin on the glycoproteins found in the cell-wall was greater than that of glycolipids and glycoproteins of the membranes (Table 3). This may occur because the inhibition of lipid and protein glycosylation often causes a decrease of protein secretion. Our data not only confirm the inhibitory effect of tunicamycin on the synthesis of lipid-linked saccharides [17], but also show that this antibiotic does not interfere with the metabolism of exogenously supplied D-[U-^{14}C]glucosamine.

Deoxynojirimycin, a glucose analogue, and deoxy-

Table 2. Amino sugar composition of radioactive endogenous pool extracted in 95% ethanol from oat coleoptile segments incubated for 3 hr in either bidistilled water (control) or tunicamycin (1 and 10 μM). D-[U-^{14}C]Glucosamine (32 kBq) was added to the incubation medium during the last 2 hr. Extracts were analysed by paper electrophoresis followed by PC. The results are from one representative experiment of three

	Radioactivity (%)		
		Tunicamycin	
Amino sugars	Control	1 μM	10 μM
D-Glucosamine	5.0	6.5	3.0
N-Acetylglucosamine	29.6	24.9	25.1
N-Acetylglucosamine 6-P	16.6	17.9	17.0
N-Acetylglucosamine 1-P	15.2	14.8	15.1
UDP-*N*-acetylglucosamine	33.6	35.9	39.8
Total radioactivity (Bq)	3722	4388	4882

mannojirimycin, a mannose analogue, are specific inhibitors of glucosidases I and II, and mannosidases, respectively. These glycosidase inhibitors operate during the processing of *N*-linked glycoproteins in plants, as well as in animal systems [17, 18]. Deoxynojirimycin at 0.5 and 5 mM stimulated the uptake of the D-[U-^{14}C]glucosamine with respect to the control by *ca* 42 and 46%, respectively. In addition, at 0.5 and 5 mM, the amount of radioactive UDP-*N*-acetylglucosamine present in the soluble pool increased by 31.8 and 41.1%, respectively (data not shown). The incorporation of radioactive *N*-acetylglucosamine into the oligosaccharide chains of membrane glycoproteins increased in the presence of deoxynojirimycin, whereas the amount of radioactive glycoproteins secreted into the walls decreased (Table 3). This contrasting effect could be explained by the incorrect glycosylation of glycoproteins, which causes a decrease of their secretion into the walls and an accumulation in the membranes. The accumulation of radioactive glycoproteins in the membranes could be also due to the lack of an alternative processing route that bypasses the glucosidase blockade. It has been reported in animal cells, that in the presence of the glucosidase inhibitors, castanospermine or 1-deoxynojirimycin, an alternative processing route that circumvents the glucosidase blockade exists. This pathway uses an endo-α-D-mannosidase, which achieves deglucosylation by hydrolysing the bond between the glucose-substituted mannose residue and the remaining polymannose units [19].

Oat coleoptile segments preincubated for 1 hr in deoxymannojirimycin at 1 and 5 mM and successively incubated for 2 hr with the same concentrations of the inhibitor plus D-[U-^{14}C]glucosamine, showed that the uptake of D-[U-^{14}C]glucosamine was not changed in the presence of 1 mM deoxymannojirimycin. Deoxymannojirimycin at 5 mM inhibited the uptake of the tracer by *ca* 30%. In spite of this, the percentage of radioactive UDP-*N*-acetylglucosamine synthesized in the presence of the glycosidase inhibitor was between 10 and 18% greater than that observed in the control segments (data not shown). Deoxymannojirimycin inhibited both the amount of radioactive glycoproteins found in the membranes and that secreted into the cell-wall (Table 3). This inhibition could be explained as a consequence of mannosidase blockade which prevents the action of other transferases, due to the lack of an appropriate substrate. Previous studies have demonstrated that, in plants, *N*-acetylglucosamine transferase I catalyzes the transfer of *N*-acetylglucosamine residues from UDP-*N*-acetylgluco-

Table 3. D-[U-^{14}C]Glucosamine incorporated into glycolipids, membrane and cell-wall glycoproteins isolated from oat coleoptile segments incubated for 3 hr in either bidistilled water (control) or different concentrations of tunicamycin, deoxynojirimycin or deoxymannojirimycin. D-[U-^{14}C]Glucosamine (32 kBq) was added to the incubation medium during the last 2 hr. The results are from one representative experiment of three

	Membrane glycolipids		Membrane glycoproteins		Cell-wall glycoproteins	
	Bq	% with respect to uptake	Bq	% with respect to uptake	Bq	% with respect to uptake
Control	28	0.43	322	4.8	632	9.4
Tunicamycin						
1.0 μM	21	0.32	262	4.0	399	6.1
10.0 μM	13	0.17	189	2.4	149	1.9
Deoxynojirimycin						
0.5 mM	40	0.42	391	4.1	296	3.1
5.0 mM	39	0.40	608	6.2	363	3.7
Deoxymannojirimycin						
1.0 mM	26	0.39	237	3.6	394	6.0
5.0 mM	19	0.41	113	2.4	273	5.8

samine to $Man_5(GlcNAc)_2$, but does not utilize $Man_9(GlcNAc)_2$ as substrate. In addition, other *N*-acetylglucosamine transferases (II–VI) could be inhibited in their action by the lack of an appropriate substrate [15].

EXPERIMENTAL

Chemicals. Pure polygalacturonase [EC 3.2.1.15] from *Aspergillus niger* was a gift from Prof. F. Cervone (Department of Plant Biology, University of Rome). Peptide *N*-glycosidase F [EC 3.2.2.18] from *Flavobacterium meningosepticum* and *N*-acetyl-β-D-glucosaminidase (*N*-acetyl-β-D-glucosaminide *N*-acetylglucosaminohydrolase [EC 3.2.1.30]) from *Diplococcus pneumoniae* were products from Boehringer. Cellulase [EC 3.2.1.4] from *Aspergillus niger*, protease [EC 3.4.21.62] from *Bacillus licheniformis* and Driselase from *Basidiomycetes* were products from Sigma.

Plant material and labelling of coleoptile segments. Subapical coleoptile segments (1 cm) were carefully prepared from 5-day-old *Avena sativa* L. seedlings (cv. Angelica) grown at 20° in the dark. Batches of 20 coleoptile segments were incubated in 1 ml of sterile bidist. H_2O containing 111 kBq of D-[U-^{14}C]glucosamine (sp. act. 7.84 GBq mmol^{-1}) for 4 hr at 20°, with constant shaking. At the end of labelling, the coleoptiles were washed rapidly in ice-cold unlabelled glucosamine and homogenized in a chilled homogeniser in 1 ml of 10 mM Na-Pi buffer, pH 7, containing 10 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF). The homogenate was centrifuged (800 *g*, 10 min, 4°) and the pellet (cell-wall material) was washed × 2 with 1 ml of homogenization buffer. Cell-wall material was incubated in 0.05% Na-deoxycholate for 2 hr at 20°, in order to remove all membrane fragments. At the end of this treatment, cell-wall material was washed with homogenization buffer (× 2) followed by Me_2CO (× 2). This fr. of purified cell-walls was used for solubilization of cell-wall glycoproteins by cell-wall-degrading enzymes and chemical extractants.

The labelling experiments in the presence of biosynthetic inhibitors and processing of *N*-linked oligosaccharide chains were performed as follows: 10 coleoptile segments were incubated at 20° for 3 hr in 700 μl of either H_2O or the appropriate soln of inhibitor (tunicamycin, deoxynojirimycin or deoxymannojirimycin) and, during the last 2 hr, 32 kBq of D-[U-^{14}C]glucosamine were added. At the end of the incubation period, segments were rapidly processed to extract the soluble pool of free amino sugars and to prepare purified cell-walls and membranes [2].

Solubilization of cell-wall glycoproteins by cell-wall-degrading enzymes. Purified cell-walls were incubated with cell-wall-degrading enzymes as follows: (a) 0.25% Driselase or 0.25% cellulase in 20 mM Na-citrate buffer, pH 5.6 at 27°; (b) 0.3 U ml^{-1} protease in 10 mM HEPES–KOH buffer, pH 8 at 37°; (c) 0.36 U ml^{-1} of polygalacturonase in 50 mM Na-citrate buffer, pH 5.3, 1 mM PMSF; at 30°. Incubations were performed in series in the presence of toluene. At the end of each incubation period (2, 4, 6, 8, 10 and 24 hr), cell-wall-degrading enzymes were inactivated at 100° for 5 min.

Solubilization of cell-wall glycoproteins by chemical extractants. Cell-wall proteins were extracted from purified walls with either 1% SDS at 95° for 15 min or PhOH–HOAc–H_2O (2:1:1) at 70° for 30 min under shaking [20]. After centrifugation (8700 *g*, 10 min), the supernatant was concd (Centricon 10, Amicon) and the cell-wall proteins pptd with 5 vols of Me_2CO at −20°. The 8700 *g* pellet (cell-walls) was hydrolysed (3 M HCl) and scanned for radioactivity.

Extraction of glycolipids. Lipid-linked monosaccharides and oligosaccharides were extracted from membranes using the method of ref [21].

Enzymic deglycosylation. Cell-wall glycoproteins were heat-denaturated at 90° for 1 min in 0.5% SDS and then incubated with either peptide *N*-glycosidase F (4 U ml^{-1}) or peptide *N*-glycosidase F (4 U ml^{-1}), in combination with *N*-acetyl-β-D-glucosaminidase (2.5 mU ml^{-1}) in 0.05 M Na-Pi buffer, pH 7.0, containing 2.5% Nonidet P40 and 0.5 mM PMSF in a final vol. of 100 μl, at 27° for 12 hr [22]. After glycosidase treatments, reaction mixts were passed through Centricon 10. The filtrate was taken to dryness, dissolved in bidist. H_2O and analysed by PC (solvent system: A) for the separation of oligosaccharide chains. Alternatively, 80% cold EtOH (final concn) was added to the reaction mixt. to ppt. protein frs. The supernatant containing *N*-glycans was collected and subjected to PC as described above.

Chemical hydrolysis. Purified cell-walls and membranes were autoclaved in 3 M HCl for 1 hr at 120°. Cell-wall glycoproteins were incubated in either 0.5 M NaOH–1 M $NaBH_4$ at 20° or 1 M NaOH–1 M $NaBH_4$ at 50°. At the end of the incubation period, hydrolysates were neutralized with HOAc and passed through Centricon 10. The filtrate was deionized on Amberlite IR-120, taken to dryness, resuspended in bidist. H_2O and electrophoresed at pH 2.0, 5 kV, 45 min. Radioactive peaks were eluted and chromatographed.

SDS-PAGE. SDS-PAGE was carried out as described in ref. [23] on a 13% polyacrylamide gel. Protein bands were stained with Coomassie Brilliant Blue R-250. Labelled proteins were detected by autoradiography on Hyperfilm-β-max (Amersham).

Paper chromatography and electrophoresis. Chromatography was performed on Whatman No. 1 or 3 MM paper in the following solvent systems: solvent A, EtOAc–pyridine–H_2O (10:4:3); solvent B, EtOH–NH_4OAc 1 M pH 3.8 (5:2); solvent C, n-BuOH–pyridine–morpholine tetraborate 0.05 M, pH 8.6 (7:5:2). Paper electrophoresis and detection of markers were performed as previously described [2].

Radioactivity counting procedure. Radioactivity on paper chromatograms and electrophoretograms was estimated as described in ref. [21].

Acknowledgement—This study was supported by a grant from MURST of Italy.

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