In Vitro Biosynthesis of 1,4-β-Galactan Attached to a Pectin-Xyloglucan Complex in Peas

By

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DECLARATION

The work reported in this thesis is my own and is original except where specific reference is made.

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ABSTRACT

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Cell-wall matrix polysaccharides are synthesised in the Golgi apparatus and then transported to the cell wall. We have investigated the properties of nascent pectin formed by the biosynthetic system *in vitro*. Particulate enzyme preparations were prepared from etiolated pea epicotyls (Baydoun *et al.*, 2001) and used to assay for 1,4-β-galactan synthase using UDP-[U-^{14}C]galactose. Optimum conditions for 1,4-β-galactan synthesis were determined. The enzyme products were characterised by selective enzymatic degradation and anion-exchange chromatography. Evidence was obtained for the formation of 1,4-β-galactan chains attached to a pectic backbone containing both polygalacturonic acid and rhamnogalacturonan I. The results also indicated that part or all of this nascent pectin was present as a complex with xyloglucan. This complex may be similar to pectin-xyloglucan complexes found in the cell wall in rose suspension cells (Thompson and Fry, 2000) and cauliflower stem (Femenia *et al.*, 1999).

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LIST OF ABBREVIATIONS

%	Percent
Bq	Bequerel
CDTA	Cyclohexane diamine tetra-acetic acid
ctrl	Control
EDTA	Ethylene diamine tetra-acetic acid
EPW	Ethyl acetate/ pyridine/water
Fuc	Fucose
Gal	Galactose
GAX	Glucuronoarabinoxylan
GDP	Guanosine diphosphate
Glc	Glucose
GS	Galactan synthase
hr(s)	Hour(s)
Kav	Kav= (Ve-Vo)/(Vt-Vo), Ve: sample elution volume, Vo: elution volume for material totally excluded from gel, Vt: total volume of packed bed
KBq	Kilobequerel
KDa	Kilodalton
KDO	3-deoxy-manno-octulosonic acid
Min	Minute
ml	Millilitre
mRNA	Messenger RNA

n	Degree of polymerisation of polysaccharide
nm	Nanometer
NMR	Nuclear magnetic resonance
PG	Polygalacturonase
PGA	Homogalacturonan (polygalactouronic acid)
PL	Pectin Lyase
PME	Pectin methyl esterase
PNP-galactoside	Para-nitrophenyl-β-D-galactoside
Rgal	Distance moved relative to distance moved by galactose marker
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
rpm	Rotation per minute
SN	Supernatant
TFA	Trifluoroacetic acid
TGN	*Trans* Golgi network
UDP	Uridine diphosphate
XG	Xyloglucan
Xyl	Xylose
v/v	Volume / volume

Chapter I

INTRODUCTION

A. The Plant Cell Wall

The plant extracellular matrix, commonly referred to as the cell wall, is a heterogeneous macromolecular assembly consisting of cellulose microfibrils embedded in a matrix of complex polysaccharides and glycoproteins (Fry, 1988; McNeil *et al.*, 1984). Knowledge of the architecture and assembly of the cell wall is of importance to be able to manipulate plant growth and morphogenesis. The cell wall is a dynamic structure whose composition and properties constantly respond to the growth, the stage of differentiation, and the environment of the cell (Northcote, 1972). In the first stage of development, the developing cell plate, or phragmoplast, is derived from vesicles of the Golgi apparatus and grows outward until it fuses with the existing primary wall (Gibeaut and Carpita, 1994). The phragmoplast is formed of callose, a (1→3)-β-D-linked homopolymer of glucose, and other polysaccharides. After cytokinesis is complete, formation of independent walls is observed as cellulose microfibrils are woven around the newly formed daughter cells (Mineyuki and Gunning, 1990). The second stage of development of the plant cell wall involves expansion and differentiation of the primary cell wall. During cell wall expansion, many co-ordinated processes are involved such as: the hydrolysis of non-cellulosic polymers, the disruption of the hemicellulose-cellulose network by proteins (example expansins), the separation of fibrillar components by osmotic pressure, and the deposition of new microfibrils or associated polymers in the innermost surface of the cell wall forming a highly stratified and cross-linked surface (Carpita and Gibeaut, 1993). Cell expansion or elongation proceeds till maturation occurs resulting in toughening of tissues. Toughening has been shown to be related to the synthesis of pectic-xylan-phenolic complexes (Waldron and Selvendran, 1992) or to embedding of other structural proteins or lignin in the cell wall

matrix, depending on the type of plant and tissue. The last stage, which is not found in all plant cells, involves the formation of secondary wall thickenings. The secondary wall is composed mostly of cellulose, lignin, and other polysaccharides such as xylans and glucomannans (Northcote, 1985).

In addition to providing a static structure to plant cells, cell walls play a role in signalling and communication between cells during adaptation and defence responses (Jarvis, 1984; Fry *et al.*, 1993). Alteration in cell wall structure can allow stem cell elongation in peas and textural changes during fruit ripening (Tucker and Grierson, 1987). The changes in fruit ripening are often most apparent in the pectic components of the wall.

Many attempts have been made to produce models of the whole cell wall and its intermolecular linkages, but due to its extreme complexity it is perhaps better to visualise the cell wall as containing a number of polymer networks, which when superimposed, interact to give rise to the whole complex structure (Brett and Waldron, 1996). The networks include: the cellulose-hemicellulose network, the pectin network, the extensin or protein network, and the lignin network. Before entering into some details of these networks, it would be easier to understand the composition of its components. The main focus will be on the first two networks and their components due to their more relevant importance in our work.

B. Cell Wall Components

1. Polysaccharides

The polysaccharides of plant cell walls are complex molecules that serve different functions. They consist of a microfibril phase and a matrix phase (Brett and Waldron, 1996).

a. Cellulose

Cellulose is the microfibrillar component of the cell wall of almost all green plants. Some exceptions exist in certain algae, where the microfibrils are composed of xylans [β (1→3) linked xylose residues] or mannans [β (1→4) linked mannose residues]. Cellulose is made of long chains of unbranched β (1→4) linked glucose residues with a degree of polymerisation between 2,000 to 6,000 residues per chain in primary cell walls, and more than 10,000 in secondary walls (Delmer, 1983). The exact arrangement and biosynthesis of cellulose is still not clear. The use of the *Acetobacter xylinum* model and the discovery of the activator of cellulose synthase (cyclic diguanylic acid) are helping in elucidating this matter (Delmer, 1999). It is known that UDP-glucose is its precursor. The appearance of terminal complexes by freeze-fracture electron microscopy suggests that cellulose is formed at or outside the plasma membrane (Delmer, 1987). It has been suggested that cellulose microfibrils may be formed by a large multienzyme complex which elongates the entire microfibril, and thus is easily disrupted causing callose or β (1→3)-glucan synthesis (Brett and Waldron, 1996). Direct evidence has recently been obtained that the terminal complexes seen by electron microscopy are involved in cellulose synthesis (Arioli *et al.*, 1998).

Complex polysaccharides constitute the bulk of the matrix molecules of cell walls and are the principle products of growing plant cells. The two major classes of complex polysaccharides are the hemicelluloses and the acidic pectic polysaccharides (Bolwell, 1988).

b. Hemicelluloses

The hemicelluloses include: xylans, glucomannans, mannans, galactomannans, glucuronomannans, xyloglucan, callose (β1,3 glucan), arabinogalactan II, and mixed (β1,3-β1,4) glucans. Of these hemicelluloses, xyloglucan is of major interest to this work.

i- Xyloglucan

Xyloglucan (XG) [Fig 1] is the predominant hemicellulosic polysaccharide of the primary cell walls of dicotyledonous plants (Fry, 1989), typically forming about 20% of the cell wall (McNeil *et al.*, 1984). This large polysaccharide (degree of polymerisation up to 2,200) is composed of a $\beta(1\rightarrow4)$-linked glucosyl backbone that is decorated at regular intervals with $\alpha(1\rightarrow6)$-linked xylose. Some of the xylose residues are substituted with galactose or fucosylgalactose units (Hayashi and Maclachlan, 1984). Digestion of most dicotyledonous xyloglucans with cellulase yields primarily the heptasaccharide and nonasaccharide repeating units and these appear to form alternating sequences (Hayashi, 1989). In some species, arabinose can be linked to a xylosyl residue of pentameric units (Mori *et al.*, 1980). Xyloglucans from monocotyledons differ from those in dicotyledons, in that the former have less xylose, less galactose, and some lack the terminal fucose (Kato and Matsuda, 1985). However, some evidence has

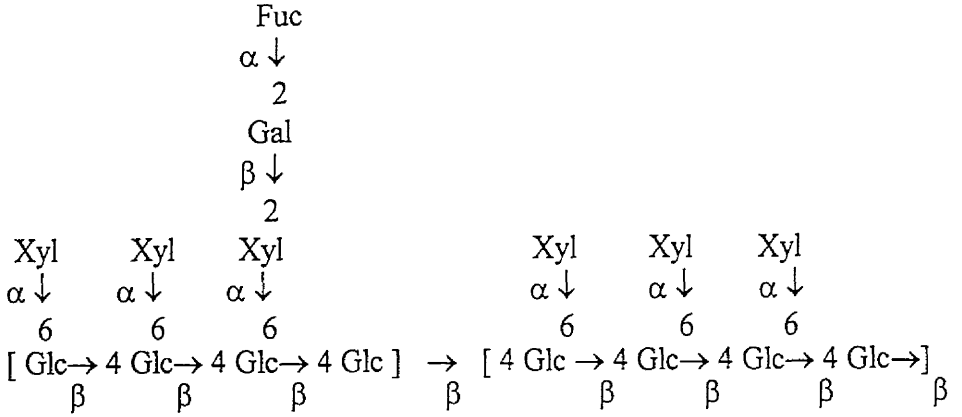

Fig 1. Chemical repeating unit of pea xyloglucan.

been provided by McDougall and Fry (1994) to show the presence of fucose in some grass cell walls.

Progress towards understanding the compartmentalisation of xyloglucan synthesis within Golgi stacks has come from an immunocytochemical study of high pressure freeze-substituted cultured sycamore cells with antibodies specific to the xyloglucan backbone and to the terminal fucose of the side-chain (Zhang and Staehelin, 1992). The synthesis of the backbone of xyloglucan was found to occur exclusively in the trans Golgi cisternae, as previously suggested by Hayashi and Matsuda (1981 a) and Moore *et al.* (1991). The labelling with the anti-terminal-fucose antibodies was consistent with the hypothesis that fucosylation of side-chains takes place in the trans Golgi cisternae and the trans-golgi-network (TGN) (Hayashi and Matsuda, 1981 b). The stimulation of xylosyltransferases by UDP-glucose to form xyloglucan in the high-density Golgi membranes, but not in other subfractions, shows that the synthesis of xyloglucan is in the denser part of the Golgi (Baydoun and Brett, 1997). On the other hand, Brummell and colleagues claimed, that synthesis was throughout the whole Golgi apparatus (Brummell *et al.*, 1990). Baydoun *et al.* (2001), claimed that xyloglucan fucosyltransferase was present in the low density Golgi membranes. These contrasting results may indicate that significant amounts of xyloglucan are synthesised the low and medium density Golgi membranes, even though the high density Golgi membranes contain many of the enzymes involved in xyloglucan synthesis. The observation that antibodies to xyloglucan bind preferentially to the trans Golgi cisternae in some tissues (Staehelin and Moore 1995) may then indicate that mature xyloglucan, much of which is synthesised in the cis- and medial-Golgi, accumulates in the trans-Golgi prior to secretion to the wall.

Xyloglucan is synthesised by the co-operative action of several glycosyltransferases and it has been shown that concurrent assembly of both glucose and xylose are obligatory (Hayashi and Matsuda, 1981 a and b). Thus xyloglucan-glucosyltransferase and xyloglucan-xylosyltransferase act in concert. But neither galactose nor fucose seems to be required for the elongation of the xyloglucan backbone (Gordon and Maclachlan, 1989). Although galactosylation precedes fucosylation, there is no co-operative linkage between the two enzymatic reactions (Camirand and Maclachlan, 1986; Farkas and Maclachlan, 1988).

Xyloglucans are thus composed of repeating heptasaccharide units (Glc_4Xyl_3), designated -XXXG-, where X is xylosylglucose and G is glucose at the reducing end (Fry *et al.*, 1993). The galactose is added to one or both of xylose units near the reducing end. It seems that xyloglucan galactosyltransferase preferentially galactosylates the nascent xyloglucan backbone at every second heptasaccharide, forming a (-XXXG.XXLG-) backbone (where L represents the galactosylated xylosylglucose) which is later fucosylated (Guillen *et al.*, 1995). The acceptor structure for xyloglucan fucosyltransferase has been shown to contain two important domains, a domain to be fucosylated, and the recognition-binding sequence that binds the enzyme (Maclachlan *et al.*, 1992). Thus, the fucosyltransferase would be expected to bind to one octasaccharide and to fucosylate the next octasaccharide two subunits away, and thus it must recognise at least three subunits of xyloglucan (Faik *et al.*, 1997).

McCann *et al.* (1992) have shown that xyloglucan molecules isolated from onion cell walls appear to be assembled from 30-nm building blocks. The xyloglucan molecule is a major structural component of cell walls. It can form hydrogen bonds to cellulose, thus forming a network [discussed later] (Talbott and Ray, 1992). In addition

to its structural role, chemically defined fragments of these polysaccharides have been shown to possess regulatory activities (Ryan, 1987). Hayashi and Delmer showed that the addition of purified pea xyloglucan to *A. xylinum* culture could prevent the fasciation of microfibrils into large ribbons. This shows that xyloglucan may play a role in limiting the size of microfibrils during cell expansion (Delmer, 1987). Also the loss of some residues from side-chains of xyloglucan may induce cell wall loosening (Reiter *et al.*, 1993).

ii- Other hemicelluloses

Other hemicelluloses such as: xylans, glucomannans, mannans, galactomannans, glucuronomannans, callose (β1,3 glucan), arabinogalactan II, and mixed (β1,3-β1,4) glucans are also included in the hemicellulosic fraction of the plant cell wall and play a role in maintaining the integrity of the plant cell wall.

Xylans form the bulk of the hemicellulose fraction of angiosperms. The general structure of xylans in higher plants is that of a main chain of D-xylopyranose residues joined by $\beta(1\rightarrow4)$ links. The degree of polymerisation is about 150-200 residues and short terminal side-chains are attached. For instance in angiosperms, one 4-O-methyl-D-glucuronic acid exists for every ten xylose residues (Timell, 1964) and it is joined by $\alpha(1\rightarrow2)$ bonds. Most of the acetylation of the xylose groups is at C-2 and C-3. In grasses, the xylans have the D-xylopyranose joined by $\beta(1\rightarrow4)$ links but the major side-chains are L-arabinofuranosyl groups attached by an $\alpha(1\rightarrow3)$ bond to the backbone, as well as 4-O-methyl-D-glucuronic acid side-chains which are $\alpha(1\rightarrow2)$ linked (Northcote, 1972). Xylans are synthesised by the co-operative action of several glycosyltransferases where UDP-xylose acts as a donor for xylose residues. From cell fractionation studies, it

appears that UDP-xylose: xylan xylosyltransferase is located in the Golgi apparatus (Waldron and Brett, 1987) mostly in the low and medium-density Golgi subfractions and to a lesser extent in the high-density Golgi membranes (Baydoun and Brett, 1997). As for their arrangement, xylan molecules seem to be oriented parallel to the cellulose chains in the plant cell wall and seem to adsorb to them (Northcote, 1972).

The mannans and galactomannans are found in some seed endosperms and seed cotyledons and function as food reserve (Brett and Waldron, 1996). The mannans consist of a linear chain of mannose residues joined by $\beta(1\rightarrow4)$ bonds and where galactose is present, it is linked as a side-chain by an $\alpha(1\rightarrow6)$ bond. UDP-galactose is transferred to the most recently transferred mannose residue (that is to the non-reducing terminal) (Edwards *et al.*, 1992). The mannans are able to form hard crystalline structures, and act as microfibrils in some algae (Brett and Waldron, 1996).

The glucomannans form the bulk of the hemicellulose fraction of secondary walls of gymnosperms. They consist of chains of randomly arranged D-glucose and D-mannose joined by $\beta(1\rightarrow4)$ linkage, the main chain may be branched once or twice. Glucomannans have a similar conformation to that of cellulose and the chains are arranged in a paracrystalline array between the cellulose microfibrils and strongly adsorb to it. The ratio galactose: glucose: mannose in the galactoglucomannans is 1:1:3, usually with a degree of polymerisation of about 100 residues, and most acetylation is at C-2 or C-3 (Timell, 1964).

The glucuronomannans are present in a wide range of plant cell walls. They have $\alpha(1\rightarrow4)$ linked mannose residues and $\beta(1\rightarrow2)$ linked glucuronic acid residues in the main chain. Side-chains include galactose $\alpha(1\rightarrow6)$ or arabinose $\alpha(1\rightarrow3)$ linked to mannose (Brett and Waldron, 1996).

Arabinogalactan II occurs as a general hemicellulose constituent of conifers such as larches. These molecules possess a highly branched galactan core, with $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linked galactose units. Arabinose residues joined by $\beta(1\rightarrow3)$ bonds are present on outer chains. Small amounts of glucuronic acid may also be present (Brett and Waldron, 1996).

Callose is made by most plant cells in response to wounding and at specific stages of wall development such as in phragmoplasts of dividing cells and in growing pollen tubes (Delmer, 1991). It is known to be made at the plasma membrane (Delmer, 1987). The substrate for callose synthase is UDP-glucose. It is a $\beta(1\rightarrow3)$-linked glucan.

The $[\beta(1\rightarrow3), \beta(1\rightarrow4)]$-glucans, also called mixed-link glucans, are uniquely found in grasses. These molecules are unbranched homopolymers of glucose containing a mixture of $(1\rightarrow4)$-β-D-glucose linear oligomers and $(1\rightarrow3)$-β-D-glucose "kinks" (Woodward *et al.*, 1985). UDP-glucose is a substrate for its synthesis (Becker *et al.*, 1995). The ratio of $(1\rightarrow3)$ to $(1\rightarrow4)$ links is between 1:2 and 1:3 and usually a single $(1\rightarrow3)$ linked residue separates sequences of two, three, or four $(1\rightarrow4)$ linked residues (Brett and Waldron, 1996).

c. Pectic Polysaccharides

Pectins are a class of complex polysaccharides found in the cell walls of higher plants. They contribute to the firmness and structure of plant tissue both as part of the primary cell wall and as the main component in the middle lamella, which is responsible for cell-to-cell adhesion (Carpita and Gibeaut, 1993). They act both as a hydrating agent and cementing material for the cellulosic network. In addition to that, pectins have a role in plant defence by releasing oligosaccharins (short sequences of $(1\rightarrow4)\alpha$-D-

galactosyluronic acid units, ten to fifteen residues) from the wall upon attack by various pathogens (Perez *et al.*, 2000). Oligosaccharins trigger defence responses by different mechanisms such as acting as a proteinase inhibitor-inducing factors or as elicitors of phytoalexins (McNeil *et al.*, 1984).

Pectic polysaccharides are composed of distinct structural domains linked together in characteristic patterns. They compromise acidic polysaccharides including homogalacturonan (PGA), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II) and neutral oligosaccharide chains such as galactans, arabinans, and arabinogalactans (which may be attached covalently to the acidic polysaccharide) (Knox *et al.*, 1990). The most abundant pectic polysaccharide is PGA/RG I, which is composed of covalently linked blocks of PGA and RG I (Fry, 1988; Zhang and Staehelin, 1992). The backbone consists of two types of domains, the PGA or homogalacturonan domains which are blocks of 15 to 70 α-1,4-linked-D-galacturonosyl residues interspersed periodically with single α-1,2-linked-L-rhamnosyl residues, and RG I domains [Fig 2] containing up to 300 repeats of alternating α-1,4 galacturonosyl-α-1,2 rhamnosyl residues (Lau *et al.*, 1985). PGA biosynthesis occurs by addition of galacturonic acid to the non-reducing end of the polymer chain (Scheller *et al.*, 1999). They have varying degrees of methyl esterification of their carboxyl groups (Tieman and Hanada, 1994). Pectins are thought to be secreted in the form of highly methylesterified polymers to the cell wall, where pectin methylesterase cleaves some of the methyl esters (Lau *et al.*, 1985). Plant pectinesterases help in the conversion of the protopectin to soluble pectin and pectate, thus are involved in the plant maturation and ripening processes (Camardella *et al.*, 2000) as well as in mechanisms that protect the plant from infection (Mangos and Haas, 1997). Several PGA chains with blocks of deesterified

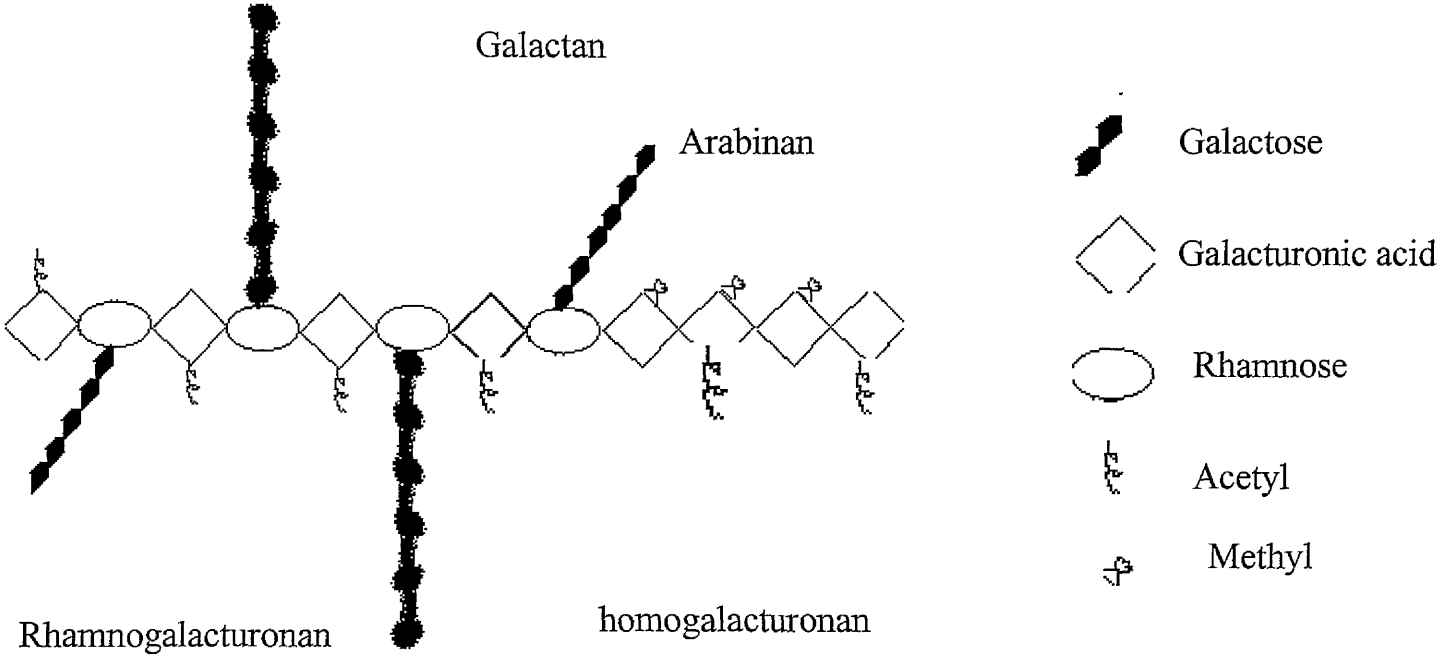

Fig 2. Rhamnogalacturonan I and homogalacturonan blocks.

galactosyluronic acid residues have been suggested to form an "egg-box" structure by forming calcium cross-bridges between chains leading to the stabilisation of the middle lamella (Demarty *et al.*, 1984; Fry, 1986). The homogalacturonan parts of the polymer (PGA) are referred to as "smooth" regions while the rhamnose-rich zones are called the "hairy" regions (Perez *et al.*, 2000). RG I is known to contain a number of different side-chains (Ishii *et al.*, 1989), mainly (1→5)-α-linked arabinose and (1→4)-β-linked galactose attached to the 4-position of rhamnose [Fig 3] (McNeil *et al.*, 1984; Brett and Waldron, 1996). These side-chains include pectic galactan, a polymer of (1→4)-β-linked galactose (discussed below).

The structural complexity of pectins suggests that there exists at least 41 unique transferases required for the direct synthesis of pectins (Doong and Mohnen, 1998). Information on the compartmental organisation of the biosynthetic pathways for PGA/RGI has come from immunocytochemical experiments with cryofixed sycamore cells (Zhang and Staehelin, 1992) and root hairs of Vicia faba L. (Sherrier and VandenBosh, 1994) using antibodies that recognise these polysaccharides. The biosynthesis of PGA/RG I appears to encompass all the Golgi stacks in plants. The backbone assembly seems to start in the cis cisternae and to continue in the medial Golgi cisternae). Baydoun *et al.* (2001) showed that galactan galactosyltransferase (galactan synthase) was present in the low density Golgi membranes though previous reports suggest that side-chains of the RGI-domains are added in the trans cisternae (Driouich *et al.*, 1993). Esterification of the PGA seems to be in the trans cisternae (Moore *et al.*, 1991; Lynch and Staehelin, 1992). Following their synthesis in the Golgi apparatus, the complex polysaccharides seem to be packaged into uncoated vesicles in the TGN for their transport to the cell wall (Driouich *et al.*, 1993).

Rhamnogalacturonan I

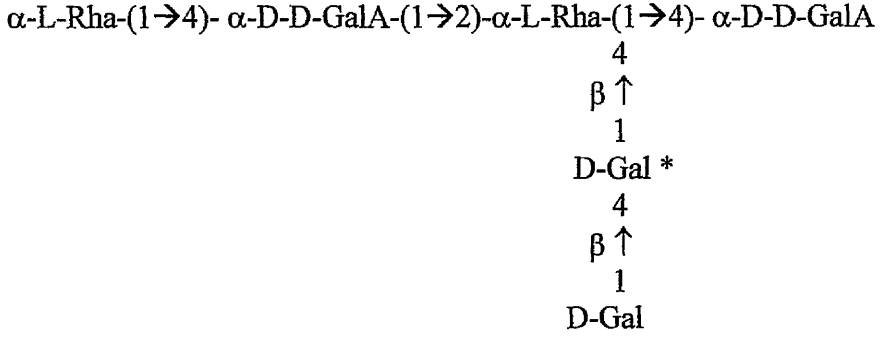

Fig 3. Chemical repeating unit of Rhamnogalacturonan I.

* Designates that different side-chains can be attached at C4 of Rhamnose (Rha): arabinans made mainly of (1→5)-linked arabinose residues, galactans made of (1→4)-linked galactose residues, or arabinogalactans. GalA designates galacturonic acid residues.

Recent work has indicated that the formation of the galactose- and arabinose-rich side-chains of pectin is developmentally regulated (Willats *et al.*, 1999; Orfila and Knox 2000). There are many indications that the galactan complex in lupin cell walls is a side-chain of a pectic rhamnogalacturonan core polysaccharide (Reid, 1985; Buckeridge and Reid, 1994) and that enzymes such as galactanases/galactosidases help in galactan mobilisation. These enzymes may be involved in turnover of primary cell walls during elongation, growth, or ripening [see section on pectin during development and fruit ripening].

Little information is available about enzymes that bring about the biosynthesis of galactans in higher plants. Panayotatos and Villemez (1973) showed that UDP-α-D-galactose is the preferred D-galactosyl donor in the formation of D-galactan chain in *Phaseolus aureus*. The presence of different galactans like $\beta(1\rightarrow4)$, $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$-galactans has been reported. $(1\rightarrow4)$-β-linked galactose attached to the OH-4 of the rhamnose of a RG I has also been shown by nuclear magnetic resonance (N.M.R.) (Davis *et al.*, 1990). Panayotatos and Villemez (1973) demonstrated the synthesis of an alkali-insoluble $\beta(1\rightarrow4)$ galactan in *Phaseolus aureus* hypocotyls. McNab *et al.* (1968) and Goubet and Morvan (1993) demonstrated the synthesis of a water-soluble galactan in *Phaseolus aureus* and flax (*Linum usitassium* L.) cells respectively. Goubet and Morvan (1993) demonstrated the presence of an alkali-soluble $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ galactans in flax and this enzyme has been further characterised by Allison and Reid (1998). Geshi *et al.* (2000) showed that the 1,4-β-galactan synthesised by membrane fractions from potato suspension cells is attached to an endogenous RG-I. Goubet *et al.* (1995) found that 1,4-β-galactans can exist either as long neutral chains or attached to RG I. Goubet and Morvan (1993 & 1994) gave evidence for the presence of two

galactosyltransferases in flax cells; at pH5 the product was found to be a $\beta(1\rightarrow3)$, $\beta(1\rightarrow6)$ galactan, while at pH8 the product was a 1,4-β-galactan. Thus there seem to be at least nine different galactosyltransferases involved in synthesising different linkages in pectin (Geshi *et al.*, 2000)

Other pectic polysaccharides are the arabinans, the arabinogalactans, and the rhamnogalacturonan II (RG II). Arabinans consist of a $\alpha(1\rightarrow5)$-linked arabinose backbone to which an $\alpha(1\rightarrow2)$ or $\alpha(1\rightarrow3)$ single arabinose side-chains are attached. Arabinogalactans consist of a backbone of $\beta(1\rightarrow4)$-linked galactan with $\alpha(1\rightarrow5)$ linked-arabinose side-chains. RG II is a complex polysaccharide of approximately 30 glycosyl residues that has a backbone of 1,4-linked-α-D-galacturonic acid (Doong *et al.*, 1995). It has complex side-chains that contain galacturonic acid, rhamnose, galactose, and some rare sugars (aceric acid, apiose, and 3-deoxy-manno-octulosonic acid (KDO).

C. The Cell Wall Networks

The cell wall is made of different networks that are superimposed. The major networks are the cellulose-hemicellulose network, the pectin network, the extensin or protein network, and the lignin network. Only the cellulose-hemicellulose and the pectin network are of main relevance to this work; thus they will be explored in more details than the rest of the networks.

1- The Protein or Extensin Network

The hydroxyproline-rich glycoprotein, extensin, forms a network within the cell wall. It is synthesised and secreted through the plasma membrane as a water-soluble

precursor protein that becomes insoluble once it is linked to the extensin network in the cell wall (Brett and Waldron, 1996). The nature of the cross-link is not certain; it may involve isodityrosine bonds between tyrosine residues of extensin molecules, tyrosine-lysine bonds between two extensin molecules, ionic bonds with pectins, or covalent or non-covalent bonds with other networks. Extensins help in maintaining the rigidity of cell wall and in stopping cell growth or elongation.

2- The Lignin Network

Lignin is an insoluble constituent of the cell wall that is aromatic, of high molecular weight, and derived by the enzymatic dehydrogenation and subsequent polymerisation of alcohols. Lignin is a hydrophobic filler material that replaces the water that was in the cell wall at early stages (Northcote, 1972). Covalent bonds can form between lignin and components of other networks to form an organised structure. The end result of lignification is the transformation of the wall from a dynamic extensible structure of the wall into a rigid structure (Brett and Waldron, 1996).

3- The Cellulose-Hemicellulose Network

Molecular architecture within the primary cell wall has been observed by microscopy (McCann *et al.*, 1990) and inferred from different enzymatic and extraction methods that emphasise the presence of cellulose-hemicellulose and pectin networks. These polymer networks were synthesised without the limitations of a cellular framework by adding xyloglucan to the fermentation media of *Acetobacter xylinus* (bacteria that synthesise cellulose extracellularly). Xyloglucan was found to form

composites with cellulose (Whitney *et al.*, 1995). Mechanical measurements (Whitney *et al.*, 1999) showed that xyloglucan-cellulose networks provide a balance of strength and extensibility that the primary cell wall needs which otherwise could not be achieved by cellulose alone.

The hemicellulosic polysaccharide xyloglucan (XG) binds with a strong affinity to cellulosic cell wall microfibrils (Levy *et al.*, 1991). Pea xyloglucan molecules, with an average molecular weight of 330,000, contain a backbone of 1,100 contiguous 1,4-linked glucose units, which represents a maximum chain length of 550nm (Hayashi and Maclachlan, 1984a). This is many times the diameter of cellulose microfibrils and quite sufficient to cross-link adjacent fibrils by H-bonding (Albersheim, 1976) even if some free sectors assumed a nonlinear configuration. An α-L-arabinosyl residue (Kiefer *et al.*, 1990), a β-xylosyl residue, and an α-L-arabinofuranosyl-(1→3)-β-xylosyl residue (Hisamatsu *et al.*, 1992) have all been found to attach at low frequency to the glycosyl residue of the glucan backbone to which is also attached the α-xylosyl residue. The extra glycosyl residues may interfere with the binding of xyloglucan (1→4) glucan backbone to cellulose (Hayashi *et al.*, 1994). Therefore, parts of the chain regions containing these residues appear to be disconnected around cellulose microfibrils and may be located in cross-linking regions (Baba *et al.*, 1994). Thus, although most models suggest covalent bonds between xyloglucan and other polymers, the chains can also entangle noncovalently (Carpita and Gibeaut, 1993). Computer modelling suggests that the fucose-galactose-xylose-side-chain of xyloglucan alters the conformation of the glucan backbone to facilitate binding to the cellulosic microfibrils (Levy *et al.*, 1991). Pea cell wall xyloglucan was found to have a greater ability to bind to cellulose microfibrils than nasturtium xyloglucan (lacking the trisaccharide side-chain). This is

consistent with the structural role of pea xyloglucan during epicotyl growth as compared with the more enzyme accessible xyloglucan during germination (Levy *et al.*, 1997). Modification of the side-chains is therefore a potential control on the properties of cellulose-xyloglucan network.

Thus xyloglucan forms a ribbon-like molecule that cross-links cellulose fibrils. The dynamic nature of this cross-linking controls the regulation of plant cell expansion and growth (Hayashi, 1989). Xyloglucan is shown to be highly susceptible to hydrolysis by endoglucanase (Hayashi and Maclachlan, 1984) and such enzymes are known to be regulated by growth hormones in many plants. Auxin leads to solubilisation and decrease of molecular weight of the polysaccharide (Hayashi *et al.*, 1984). An α-fucosidase enzyme seems to be also involved in the modification of xyloglucan due to auxin stimulation (Farkas *et al.*, 1991). Thus, other than auxin-induced turnover of xyloglucan (Labavitch and Ray, 1974 a & b), xyloglucan is also degraded due to acid growth (Jacobs and Ray, 1975; Terry *et al.*, 1981). However, feedback inhibitors of auxin stimulated growth and xyloglucan degradation exist to maintain the cell wall integrity. These inhibitors are fragments of the xyloglucan itself after degradation by endoglucanase as the xyloglucan nonasaccharide fragment (York *et al.*, 1984).

In grasses, a similar network exists which is also a hemicellulosic-cellulose network. But the hemicellulose in this case is glucuronoarabinoxylan (GAX) (Brett and Waldron, 1996). Thus, the cellulose microfibrils provide the mechanical strength for the cell or tissue and are usually coated with tightly bound hemicelluloses (XG or GAX). Other XG or GAX probably with altered fine structures and physical properties form a continuum with pectins in the plant cell wall (Carpita and Gibeaut, 1993).

4- The Pectin Network

Some pectins can be extracted with hot water (Goldberg *et al.*, 1989; Goubet and Morvan, 1993), with cold water after hot ethanol pre-treatment (Carrington *et al.*, 1993), or by the addition of a chelating agent such as EDTA (Davis *et al.*, 1990) or CDTA (cyclohexanediamine tetra-acetic acid) [Carrington *et al.*, 1993; Rihouey *et al.*, 1995], without altering the appearance of the cellulose-hemicellulose network. Pectins may be connected to other cell wall components or other pectins via ionic or covalent cross-links (Stolle-Smits *et al.*, 1999). Pectins interact in the wall in many ways. First, the helical chains of PGAs can condense by cross-linking with Ca^{+2} (in ionic bonds) to form junction zones, linking several chains together and forming a gel (McCann and Roberts, 1994). Pectins may be cross-linked further via ester linkages with dihydroxy cinnamic acids, such as diferulic acid, to form covalent attachments with other polymers. A large part of the covalent glycosidic binding in pectins is via the neutral side-chains (arabinosyl or galactosyl) since their loss increases the ability of pectins to slide across other molecules or to be more accessible to enzymes such as pectin methylesterase (PME) or polygalacturonase (PG) (Thakur *et al.*, 1997). The size of the junction zones and the size and frequency of polymer substitution on RG, and the high level of neutral sugar side-chains (Foster *et al.*, 1996) can constitute a fine control of wall porosity, matrix charge, ion balance, and modulation of pH (Carpita and Gibeaut, 1993). Energy calculations show that the galactan side-chain has intermediate stiffness (Duda *et al.*, 1991), hence it can allow the strength of polymers such as cellulose to be used efficiently (Girault *et al.*, 1997). These side-chains can ensure that cracks or stress propagate along the matrix instead of across the fibres. Also galactans can decrease the ability of pectin molecules to cross-link and form a coherent gel network. Thus

extensive cross-linking exists in epidermal cells of tomato fruit and collenchyma cell wall thickenings where acidic pectic epitopes are abundant and neutral side-chains are absent (Ha *et al.*, 1996).

Another common modification of the pectin polymers is esterification of the carboxylic acid functional groups. Elongation is correlated with increased esterification and the cessation of elongation with de-esterification (McCann and Roberts, 1994). Pectins can also serve more subtle roles as recognition molecules that signal appropriate developmental responses to symbiotic organisms, pathogens, and herbivores (Carpita and Gibeaut, 1993).

5-Interactions between XG-cellulose and Pectin Networks:

Pectin and cellulose are major components of most primary cell walls, yet very little is known about how they interact either during assembly or other processes of the cell wall (Chanliaud and Gidley, 1999).

Recent models for primary plant cell wall structure emphasise co-extensive networks of pectin and cellulose-hemicellulose (Carpita and Gibeaut, 1993; Talbott and Ray, 1992). These models suggest that there exists two "entangled but distinct polymer networks" a xyloglucan-cellulose network, held together by hydrogen bonds, and a pectin network part of which is held together by calcium bridges (McCann and Roberts, 1991) or other bonds (see section on Pectins). These models stress that no bonds exist between the pectin and cellulose networks, but rather that they act independently [Fig 4]. This view is supported by experiments that show release of pectins from cell walls via sequential extraction with chelating agents and alkali before the release of hemicelluloses. But there is evidence that some pectin resists such extraction and

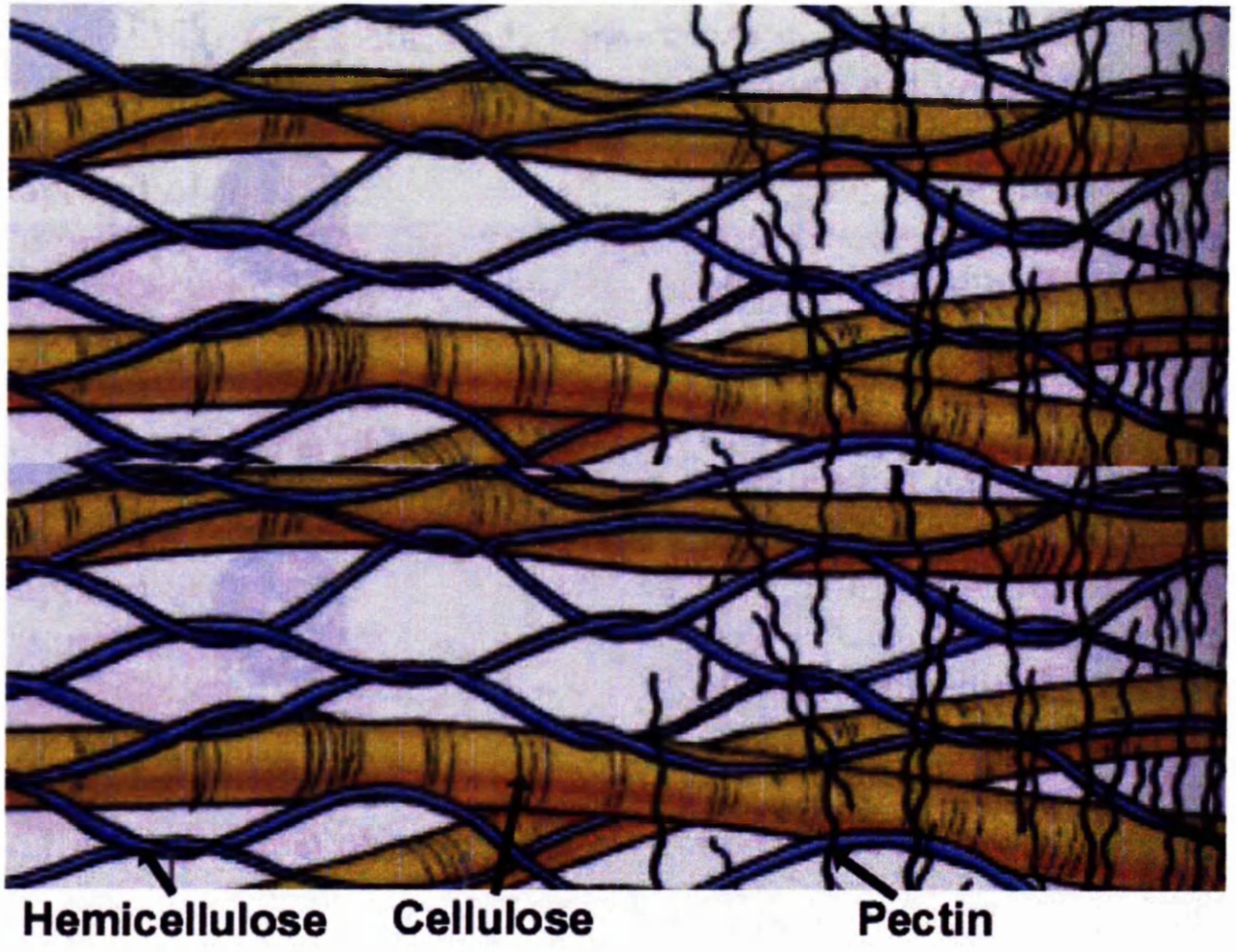

Fig 4. Independent hemicellulose and pectin networks in the plant cell wall. Modified from Carpita and Gibeaut,1993.

remains attached to the cell wall (Chanliaud and Gidley, 1999). Additional evidence for independent networks was provided by experiments where pectin polymers where added at the point of synthesis of cellulose of the bacterium *Acetobacter xylinus* (in the fermentation medium) to mimic the cell wall deposition phenomena (Chanliaud and Gidley, 1999). This work gave no evidence for the incorporation of pectin within the cellulose fibrils (both by NMR and CDTA extractions). However the pectin used in this work is rich in homogalacturonans which are characteristic of the middle lamella where PGA molecules bind to each other via calcium bridges and cellulose is deficient. Interestingly this work also showed that the presence of pectin at the time of cellulose deposition had an effect on the extensibility without compromising the strength. Also, Shedletzky *et al.*(1990) showed the ability of plants to grow on 2,6-dichlorobenzonitrile, a herbicide that inhibits cellulose biosynthesis. In such a system the XG-cellulose network is absent and wall have a higher proportion of homogalacturonan and rhamnogalacturonan polymers. The flexibility of plant cells shown here gives evidence that one network can be present without the other. Wells *et al.* (1994) gave evidence by high-resolution images that the cell wall of tomatoes is constructed from at least two independent networks (pectin and hemicellulose-cellulose networks) in the primary cell walls. Neither the spacing of the pectin molecules nor the thickness of the cell wall was affected with the reduction in the hemicellulose-cellulose network.

Opposing models (Keegstra *et al.*, 1973) propose that XG, pectic polysaccharides, and glycoproteins are all linked together via covalent bonds. The nature of the covalent bonds between xyloglucan and acidic pectins is not yet elucidated. Since it is stable (after alkali treatment) then it does not seem to be through

ester nor O-glycosidic through serine or threonine. It could be a glycosidic linkage between the reducing end of xyloglucan and the non-reducing sugar end of pectin (Keegstra *et al.*, 1973). The converse is not possible if the linkage is to involve a galactan or arabinogalactan side-chain (Thompson and Fry, 2000) of acidic pectin [Fig 5]. Coimbra *et al.* (1995) gave further evidence for the covalent linkages between networks by showing the presence of pectic-xylan complexes. This was also postulated by Waldron and Selvendran (1992) from work on asparagus stems during maturation. Hence interpolymeric cross-linking occurs between xylan-xyloglucan and xylan-pectic complexes. Femennia *et al.* (1999) gave good evidence by ion-exchange chromatography and other techniques of the occurrence of associated acidic xylans, xyloglucans, and pectic polysaccharides. The bonds are mostly covalent. There seems to be a maturation-related increase in cell wall cross-linking in the cauliflower stems.

Binding of nascent glucuronoarabinoxylan (Brett *et al.*, 1997) or glucuronoxylan to hemicelluloses was found to be pH-dependent and this dependency was abolished by pre-treatment with protease enzyme (Crosthwaite *et al.*, 1994). A 36-45 KDa protein was found to be strongly bound to glucuronoxylan, probably covalently. Rizk *et al.* (2000), showed that pectin was also synthesised attached to a protein that is also involved in the pH-dependent non-covalent binding of pectin to xyloglucan. The binding at low pH suggests that binding to xyloglucan does not occur in the vesicles (where the pH is neutral) but near the growing cell wall (where the pH is acidic). These proteins were named "assemblins" due to their suggested role in cell-wall assembly, helping the newly formed polysaccharides to interact with each other and with cellulose to form the innermost cell wall layer.

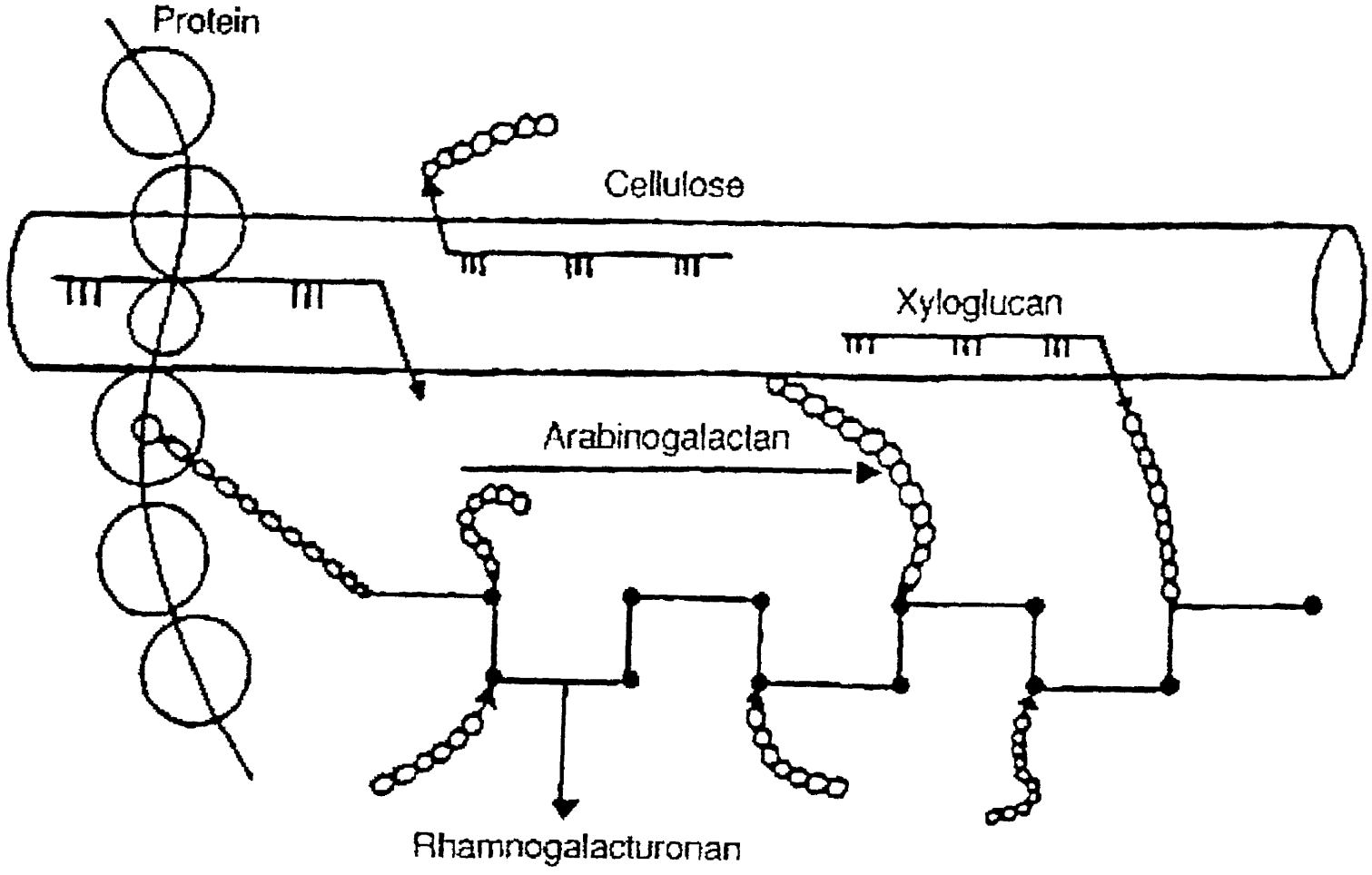

Fig 5. Molecular structure of the plant cell wall (Keegstra *et al.*, 1973).

Thompson and Fry (2000) gave evidence (by anion-exchange and enzyme treatment techniques) that around 30% of the xyloglucan in the cell walls of suspension-cultured rose cells is covalently linked to acidic pectins, whereas the rest of xyloglucan is neutral and not bound to pectins. This made them suggest an intermediate model from those suggested before where about two thirds of xyloglucan is bound to cellulose microfibrils via hydrogen bonds (Fry 1989a) but free from pectin network whereas the rest of xyloglucan are covalently linked to acidic pectins (rhamnogalacturonans) mostly probably via arabinan/galactan domains [Fig 6]. Some covalent bonds may also exist between the XG-cellulose and calcium-bridged pectins (homogalacturonans) to interlock the whole complex. But more work should be done on that.

D. Pectins during Development and Fruit Ripening

In plant cell wall, the pectin network is known to be modified in response to different developmental and environmental cues such as during cell elongation, fruit ripening, or even in response to pathogens. Immunoprofiling and immunolocalization techniques can be used to provide us with some biochemical information on the localisation of specific pectin components. Willats and Knox (1999) found that all organs of pea seedlings were labelled with JIM 5 antibody, indicating the presence of poorly methylated homogalacturonan (both highly branched and unbranched) throughout germination. Whereas in tomato, there seems to be a shift from highly branched pectin to unbranched homogalacturonan in pericarp tissue of green and red fruit. This reflects the idea that major cell wall modifications occur during ripening.

During fruit ripening, cell wall pectins are subjected to numerous modifications. First, pectins are secreted in a methylesterified form to the cell wall. Pectin methylesterase (PME) can then deesterify the pectins and they thus become available

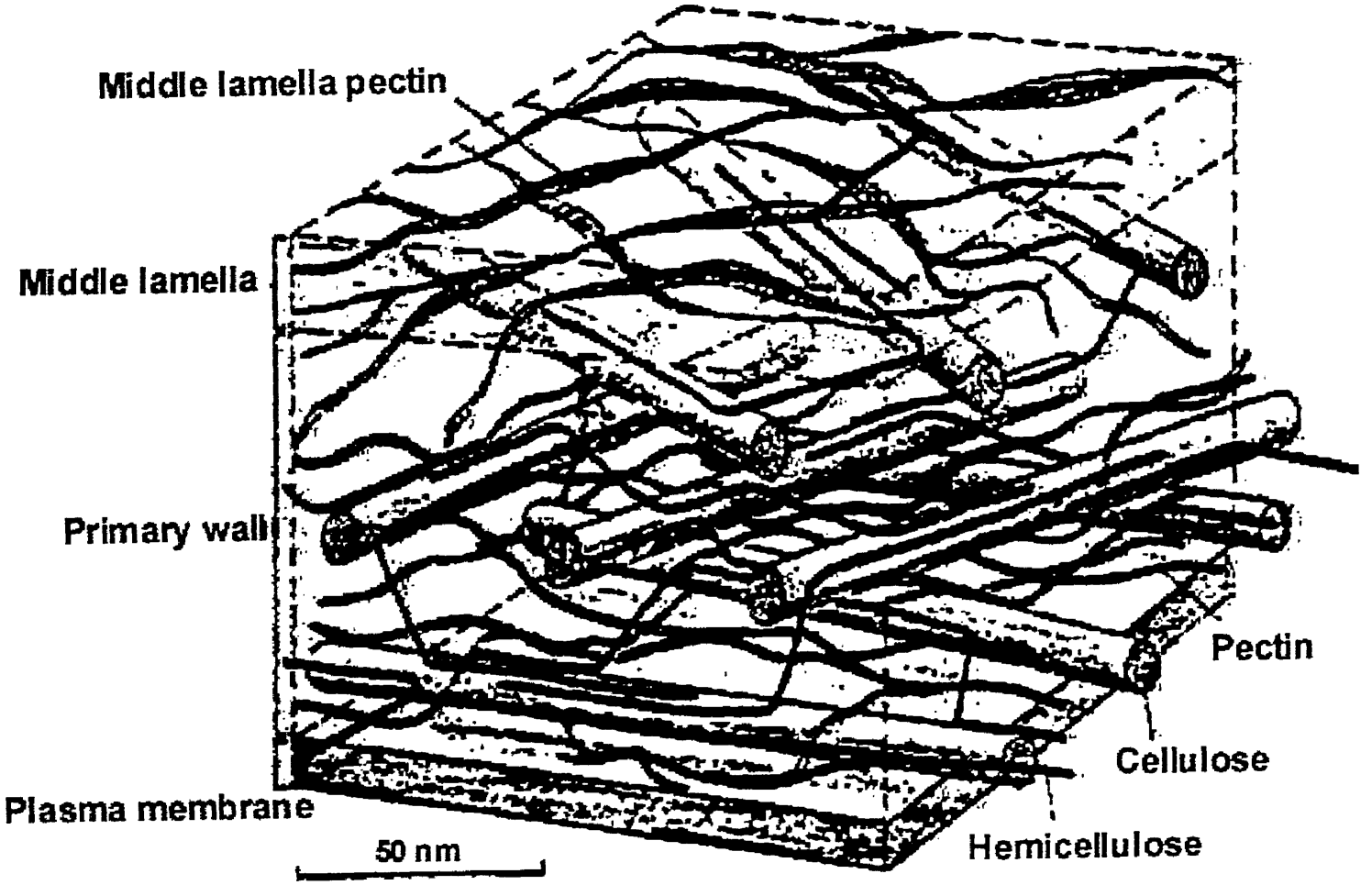

Fig 6. Primary cell wall model (McCann and Roberts, 1991) .

for calcium-mediated intermolecular cross-linkages (Carpita and Gibeaut, 1993). The degree of pectic esterification drops from 90% in immature green tomato fruit to 30% during red-ripening stage (Steele *et al.*, 1997). As ripening progresses, unesterified polyuronides are depolymerised (Smith *et al.*, 1990; Huber and O'Donoghue, 1993) and more polyuronides become susceptible to solubilisation by chelators (Seymour *et al.*, 1987a; Giovannoni *et al.*, 1989). In addition, there is a decrease in the content of neutral sugar residues, particularly galactose, associated with polyuronides as side-chains (Gross, 1984). Solubilisation of galactan has been demonstrated to be a general feature of fruit ripening such as tomato, mango, apple, and kiwi (Seymour *et al.*, 1990). In nectarines, a decreased degradation of galactan side-chains was associated with the development of mealy fruits (Dawson *et al.*, 1992). In tomato ripening, at least part of the polyuronide depolymerisation and solubilisation is due to the activity of endo-acting-polygalacturonase (Brady, 1987; Fischer and Bennett, 1991). Tomato fruit ripening and softening are accompanied by massive increases in endo-polygalacturonase mRNA abundance, immunologically detectable protein and enzyme activity (Grierson and Tucker, 1983; DellaPenna *et al.*, 1986). This is concomitant with the increase in cell wall pectin solubilisation and a decrease in polyuronide degree of polymerisation. The precise contribution of cell wall disassembly to tomato fruit softening remains unclear. It was found by Brummell and Labavitch (1997) that tomato fruits where endopolygalacturonase activity had been suppressed to less than 1% of wild-type levels were slightly firmer than in nontransgenic controls later in ripening. But this enhanced fruit firmness may be due to reduced pectin depolymerisation rather than altered extractability. Carrington *et al.* (1993), found that the loss of galactose which is evident

in both antisense fruit (containing an antisense gene for polygalacturonase) and wild type fruit is too large to be explained simply by polygalacturonase solubilisation of a rhamnose-arabinose-galactose containing polymer. Other mechanisms are clearly involved. It seems that a β-galactosidase capable of hydrolysing a β-1-4-galactan is present in tomatoes and its activity increases during ripening.

More correlation of galactan with firmness has been observed by studies done by Jones *et al.* (1997) on tomato fruit. These studies show that (1→4)-β-galactan is abundant in the pericarp of green tomato whereas it is absent from the locular gel (thus the different texture). Loss of galactose from kiwi or tomato fruit plays a role in cell wall changes that lead to fruit softening (Jones *et al.*, 1997; Redgwell *et al.*, 1997). Studies done on transgenic walls of potato plants (expressing an endo-1,4-β-D-galactanase) indicate that the reduced content of galactan in RG I results in a more porous wall architecture. Pectic material seems to be more accessible to PME and PG enzymes compared with wild-type walls. This may indicate a particular role of galactans in wall structure (Sorensen *et al.*, 2000). A strict molecular control of pectin (RG I, HG, galactan side-chains, etc.) synthesis and degradation is suggested by biochemical and immunocytochemical work on aspen. It seems that high pectic content characterises actively dividing or elongating cells. Galactan synthesis seems to occur throughout cambial zone and in enlarging xylem whereas galactan disappears progressively during differentiation of phloem (Ermel *et al.*, 2000).

McCartney *et al.* (2000), showed that there is an appearance of (1→4)-β-galactan as side-chains for RG I in pea cotyledon cell walls late in development but before seed maturation and dehydration. They found that spatial differences exist within cell walls in

the location of (1→4)-β-galactan and (1→5)-α-L-arabinan components, emphasising the complexity of RG I and the role its side-chains play. Galactans seem to have a role in increasing firmness as well as a storage role in pea cotyledons. Recent studies on lupin cotyledons, where there is a drop in galactose during germination, show that galactans have a dual role both as a reserve polysaccharide and as a molecular restraint to expansion of cotyledons during expansion [by retarding the action of pectinases on RG I, for example] (Buckeridge *et al.*, 2000). Galactan rapidly accumulated in cell walls following induction and before visible elongation (Willats and Steele-King, 1999) whereas they occurred at very low level in proliferating cell walls of carrot cells. Pectic galactans are degraded not only in ripening fruit but also in senescent flower petals and during hypocotyl extension growth (Jones *et al.*, 1997). The study done by Stolle-Smits (1999) suggests that there is a constant synthesis and degradation of cell wall material during pod elongation with a shift from neutral, sugar-rich, branched pectin (RG) to the synthesis of non-branched homogalacturonans. It seems that the pectic side-chains are more flexible than the pectic backbone hence involved in contributing to porosity of the cell wall. These side-chains may influence access of modifying proteins such as enzymes like PG or PME (Willats and King, 1999) or proteins such as expansins and xyloglucan endotransglycosylase that can alter the cellulose-hemicellulose network, thus facilitating cell enlargement (Cosgrove, 1997).

E. Aim of the Study

Little information is available about the biosynthesis of galactans in higher plants. It is known that UDP-gal may serve a precursor (Goubet and Morvan, 1993) for galactan among other products with the help of galactosyltransferase enzyme. Thus it is necessary to differentiate galactan from other products by endo –1, 4-β-galactanase digestion or other methods and to find the optimal condition of the galactan synthase enzyme in particular, instead of galactosyltransferases in general. Also the structural features of the synthesised galactan and how they are attached to the cell wall have not been investigated in detail.

Thus the objectives of this study are:

1- To optimise the conditions for galactan synthase (GS) activity in peas and to study the nature of exogenous and endogenous acceptors for galactan biosynthesis.

2- To study the nature of the product formed in vitro.

3- To study the attachment to different polysaccharides and the type of bonds involved.

4- To study the binding of the synthesised product to the cell wall or to the xyloglucan extracted from the third internodes of pea stems.

Chapter II

Materials and Methods

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MATERIALS AND METHODS

A. Chemicals

Uridine-diphospho-D-[U-^{14}C]-galactose, uridine-diphospho-D-[U-^{14}C]-xylose, guanosine-diphospho-D-[U-^{14}C]-fucose, and uridine-diphospho-D-[U-^{14}C]-glucuronic acid were purchased from The Radiochemical Centre, Amsherham, U.K. Non-radioactive sugar nucleotides (uridine diphospho-D-xylose, uridine diphospho-D-glucose, and uridine diphospho-D-galactose), bacterial protease (P-5130), fungal proteinase K (P-8044), pectin methyl esterase (P-5400), pectin lyase (P-2804), and p-nitrophenyl-β-O-galactopyranoside (N1252) were purchased from Sigma, USA.

Bio-Gel P10 and EconoPac Q cartridges were obtained from Bio-Rad Laboratories, USA. Sepharose CL-6B was purchased from Pharmacia, Sweden. Endo-1,4-β-galactanase (EGALN), endo-1,4-β-glucanase or cellulase (E-CELTR), endo-1,4-polygalacturonase (E-PGALS), xylanase (E-XYTR1) and pectic galactan were purchased from Megazyme, Ireland. Ultima-Flo AF, a biodegradable scintillation fluid, was purchased from Packard Instrument company, Meriden, CT 06450. Rhamnogalacturonase A and B were kindly given by Dr Kirk Schnorr, Novozymes A/S, Bagsvaerd, Denmark.

All other chemicals used in this work were of analytical grade. All aqueous solutions were made in glass distilled water.

B. Plant Material

Peas (*Pisum Sativum* L. cv Meteor) were obtained from Sharpes International, Sleaford, U.K. or from Thomas Dagg & Sons Ltd, 16 Bath St, Glasgow, UK. Seedlings were soaked overnight in water at room temperature and then grown on damp vermiculite for 6 days (for particulate membrane preparation) or 9 days (for xyloglucan preparation) at 25°C in continuous darkness.

C. Particulate Membrane Preparation

Membranes were prepared according to the methods described by Hobbs *et al.* (1991) and Baydoun *et al.* (2001) with minor modifications. Etiolated seedlings were used to obtain the epicotyls. The hooks were excised and discarded, while the remaining part of the epicotyls was cooled on ice. All subsequent operations were carried out at 0-4°C. The epicotyls (100 grams) were chopped with a razor blade and then homogenised using a pestle and mortar in 35 ml of a homogenisation buffer that contained 10mmol.l^{-1} Tris-HCl (pH 7.4), 10mmol.l^{-1} KCl, 1.5mmol.l^{-1} $MgCl_2$, and 10mmol.l^{-1} dithiothreitol. The homogenate was strained through four crossed layers of muslin. The residue was re-homogenised in 50 ml of homogenisation buffer and strained. The two filtrates were combined and centrifuged at 27,000g (or as indicated as initial centrifugation) for 10 minutes in a Sorvall RC-5B centrifuge. The pellet was discarded, whereas the supernatant was centrifuged at 100,000*g* for 1 hour in a Sorvall OTD-65B ultracentrifuge using an AH629 swing-out rotor. The resulting pellets were then resuspended in resuspension buffer (50mmol.l^{-1} Mes, pH5.5 or as indicated) and subjected to ten strokes in a glass-

teflon, 1ml tissue homogenizer to make the particulate enzyme preparation. This was kept on ice and used within 20 minutes.

D. Galactan synthase assay and particulate enzyme preparation

Standard incubations, (unless otherwise specified), for galactan synthesis contained UDP-[U-^{14}C]galactose (1.4 KBq, 1.4μmol.l^{-1}), $MnCl_2$ (10mmol.l^{-1}) and the particulate membrane preparation (50μl) in a total volume of 100μl. Non-radioactive uridine-diphospho-galactose (UDP-galactose) 0.2mmol.l^{-1} was added to the standard incubations in later sections (after optimisation). The assays were carried out at 25°C for 1 hour, and terminated by the addition of 90% (v/v) ethanol (1ml). The pellet was then washed three times with 70% ethanol (1ml) and once with water. The pellet was either resuspended in 0.4ml of water and its radioactivity determined by liquid scintillation counting (particulate product) or treated with a specific enzyme. To determine the incorporation into galactan, the pellets were treated with 10 units of galactanase enzyme (50ul enzyme + 150ul of 50mmol.l^{-1} sodium acetate buffer pH 4.5) or with 200ul of the same buffer (control) at 40°C for 18 hours or as indicated. The radioactivity in the solubilised material was determined.

E. Preparation of solubilised radioactive polysaccharides for analysis

For preparation of [^{14}C]-galactan, the above procedure was scaled up where appropriate. After the pellets had been washed with 70% ethanol and water, pectins were extracted by boiling for 10 minutes with 50mmol.l^{-1} EDTA-phosphate buffer (pH 6.8) and the extracts were passed through a (1 x 15cm) Bio-Gel P10 column. Where indicated,

4% KOH/0.1% $NaBH_4$ extractions were carried out at 25°C for 1 hr after the EDTA-phosphate extractions. The alkali-soluble fraction was neutralised with acetic acid, then passed through the same column.

For preparation of [^{14}C]-xylose- and [^{14}C]-fucose-labelled polymers, modifications of the conditions of Camirand *et al.* (1987) and Baydoun *et al.* (2001) were used respectively. The particulate membrane preparation was incubated with either UDP-[U-^{14}C]xylose (1.8 kBq, 1.8μmol.l^{-1}) or GDP-[U-^{14}C]fucose (1.4 kBq,1.2μmol.l^{-1}), together with non-radioactive UDP-xylose (0.02mmol.l^{-1}), UDP-galactose (0.02mmol.l^{-1}), UDP-glucose (2mmol.l^{-1}) and $MnCl_2$ (10mmol.l^{-1}), and the pellets then washed and extracted as those of [^{14}C]-galactan.

For preparation of [^{14}C]-pectin using UDP-[U-^{14}C]-glucuronic acid, referred to as "radioactive pectin", conditions of Rizk *et al.* (2000) were applied. Thus the particulate membrane preparation (191μl) was incubated with UDP-[U-^{14}C]-glucuronic acid (3.53 kBq, 1.7 μmol.l^{-1}), UDP-xylose (1mmol.l^{-1}) and $MnCl_2$ (10mmol.l^{-1}) in a total volume of 200μl at 25° C, for 4 hours. The pellets were then washed and extracted as above, but the EDTA-phosphate extracts were passed through Sephadex G-100 column.

F. Enzyme Treatments

Particulate enzyme preparations, EDTA extractions, KOH extractions, or others were incubated with the 10 units (per total 200μl incubation) of each of the following enzymes at the pH and temperature specified by the Sigma or Megazyme catalogue. Rhamnogalacturonase incubations were carried out using conditions similar to those described by Geshi *et al.* (2000). Reactions were stopped by boiling for 15 minutes at

100°C. Where required, the solubilised supernatant was neutralised and rotoevaporated before running on gel filtration columns or using in binding experiments. Control incubations were processed as above with the appropriate buffer in the absence of the enzyme. The rhamnogalacturonase and the endo-1,4-β-D-galactanase were highly purified enzymes and had no significant contamination with other activities (Geshi *et al.*,2000.

-*Endo-1,4-β-D-galactanase (EGALN):* from *Aspergillus niger* (Megazyme), 10 units in 50mmol.l^{-1} sodium acetate buffer, pH 4.5 at 40°C for 18 hours or as indicated.

-*Endo-1,4-β-glucanase or cellulase (E-CELTR):* from *Trichoderma longibrachiatum* (Megazyme), 10 units in 50mmol.l^{-1} sodium acetate buffer, pH 4.5 at 40°C for 18 hours or as indicated.

-*Pectin Lyase (P-2804) :* from *Aspergillus japonicas* (Sigma), 10 units in 50mmol.l^{-1} sodium acetate buffer, pH 5 at 40°C for 18 hours or as indicated.

-*Endo-1,4-polygalacturonase (E-PGALS):* from Megazyme, 10 units in 50mmol.l^{-1} sodium acetate buffer, pH 4 at 40°C for 18 hours or as indicated.

- *Rhamnogalacturonase:* kindly provided by Dr Kirk Schnorr, Novozymes A/S, Bagsvaerd, Denmark. 10 units in 50mmol.l^{-1} sodium acetate (pH 3.5) buffer, at 30°C for 2 hours or as indicated.

- *Proteinase K [fungal] (P-8044):* from *Tritirachium album* (Sigma), 10 units in 50mmol.l^{-1} Tris-HCl (pH 7.4) buffer, at 37°C for 18 hours or as indicated.

- *Protease [bacterial] (P-5130):* from *Streptococcus griseus* (Sigma), 10 units in 50mmol.l^{-1} Tris-HCl (pH 7.4) buffer, at 37°C for 18 hours or as indicated.

-*Pectin methyl esterase (P-5400)*: from Sigma, 10 units in 50mmol.l^{-1} Tris-HCl (pH 7.4) buffer, at 30°C for 18 hours or as indicated.

-*Xylanase (E-XYTR1)*: from *Trichoderma viride* (Megazyme), 10 units in 50mmol.l^{-1} sodium acetate buffer, pH 4.5 at 40°C for 18 hours or as indicated.

G. Gel Permeation Chromatography (Gel Filtration)

Supernatants obtained from EDTA extractions or from enzyme treatments were applied to (1 x 15cm) columns packed with Bio-Gel P10, Sepharose CL-6B, or Sephadex G-100 and were eluted with water (or as indicated). Blue Dextran and $CoCl_2$ were used as the high and low molecular weight markers respectively. Samples (0.5-1ml) were collected using a fraction collector and assayed for their radioactivity. Where indicated, the material eluted in the void volume (with the high molecular weight marker) referred to as the "high molecular weight extract" was collected for further treatment.

In some sections the galactanase enzyme was referred to as desalted. This indicates that the enzyme was run on a small Bio-Gel P4 column (1x8cm) and the high molecular weight material was concentrated and used as desalted enzyme. This procedure was carried out to remove the 3.2 mol.l^{-1} ammonium sulphate in which the enzyme is supplied. But it should be noted that much of its activity is lost due to such treatments.

H. Anion-exchange chromatography

An EconoPac High Q cartridge [5ml] (from Bio-Rad) was used for anion exchange (Willats *et al.*, 1999; McCartney *et al.*, 2000). The cartridge was eluted successively with 0, 0.3, 0.5, and 1mol.l^{-1} NaCl in 0.2mol.l^{-1} Tris (pH7.8) buffer. The

material eluted in the 0mol.l^{-1} NaCl / 0.2mol.l^{-1} Tris buffer represents the neutral fractions. The remaining fractions constitute the acidic fractions. 50 mmol.l^{-1} EDTA or 8mol.l^{-1} urea were added to the eluent where indicated. Sodium azide (0.02 %) was added to the buffers to avoid bacterial contamination.

I. Total Acid Hydrolysis

Galactosylated products were hydrolysed with 2mol.l^{-1} trifluoroacetic acid (TFA) for 1 hour in a sealed tube at 120°C as described by Baydoun *et al.* (1989). Insoluble material was removed by centrifugation at 14,000g for 10 minutes in an MSE microcentaur microfuge. Samples were rotoevaporated to dryness to get rid of all TFA, dissolved in about 50µl water and applied to a Whatman No1 paper chromatogram (or added to particulate enzyme preparations). Descending paper chromatography was carried out for 18 hours (or as indicated) in ethyl acetate/ pyridine/ water (EPW) 8:2:1 (v/v) or in EPW 10:4:3 (v/v). Radioactivity on the chromatogram was analysed by electronic autoradiography using a Packard Instant Imager or by cutting the paper into 1x4cm strips of the paper chromatogram and placing them in scintillation vials containing 1ml of scintillation fluid (Harris and Northcote, 1970). Marker sugars (glucose, galactose, arabinose or others) that were run parallel to the hydrolysed radioactive material, were detected by dipping the paper for 1-2 minutes in a solution that contained $AgNO_3$ (0.2g) dissolved in water (0.4ml), and 26ml of acetone. After drying for 3-5 minutes, it was dipped in a solution that consisted of NaOH (10mol.l^{-1}, 1.25ml) and absolute ethanol (100ml). Brown spots appeared, indicating the presence of the marker sugars (Fry, 1988).

J. Partial Acid Hydrolysis

Galactosylated products were hydrolysed with 0.1mol.l^{-1} trifluoroacetic acid (TFA) at 100°C for 3 hours, followed by descending paper chromatography in ethyl acetate/pyridine/water 10:4:3 (Panayotatos and Villemez, 1973). Galactobiose and galacto-oligosaccharide standards were generated by partial acid hydrolysis of 1,4-β-galactan.

K. Cell Wall Preparation

Cell wall preparations were based on the method described by Brett *et al.* (1997). Epicotyls from 6 days old peas were chopped using a razor blade as described in "Particulate Membrane Preparation" section and were homogenised in pestle and mortar with an equal weight of 10mmol.l^{-1} oxalic acid/10mmol.l^{-1} sodium phosphate buffer (pH 5). The homogenate was strained through two layers of muslin and then divided into 1.5ml fractions. These fractions were next centrifuged in an MSE microcentaur microfuge at 3000*g* or 6500 rpm for 5 minutes. The pellets were next washed with 0.5ml of the same buffer before they were used in binding assays.

L. Xyloglucan Preparation

Xyloglucan preparation from peas was based on modifications of the methods of Hayashi and Maclachlan (1984) and of Ogawa *et al.* (1990). Second and third internodes (50g) were harvested from 8-9 days old peas and extracted 3 times with 70% (v/v) ethanol (150ml) for 30 minutes at 70°C to remove the lipid. The tissue was then chopped with a razor blade, homogenised using a pestle and mortar in Tris-HCl buffer (0.1mol.l^{-1},

pH 7.0, 75ml), and centrifuged (8000*g*, 10 minutes). The pellets were extracted 3 times with EDTA buffer (0.1mol.l^{-1}, pH 7.0, 75ml) for 30 minutes at 85°C to remove the pectic fraction. The pellets were next extracted 3 times with 4% KOH/0.1% $NaBH_4$ (75ml) for 1 hour at 25°C in a shaking incubator; the 4% alkali removes some of the hemicelluloses present, especially xylans. Centrifugation at 8000*g* for 10 minutes resulted in an insoluble material referred to as "cell wall ghosts" consisting mainly of cellulose and xyloglucan. The pellets were next extracted twice with 24 % KOH/0.1% $NaBH_4$ (25ml) for 4 hours at 25°C in the same incubator. The 24% KOH-soluble fractions were combined and neutralised with acetic acid. The neutralised fractions were treated consecutively with salivary α-amylase (Rizk *et al.*, 2000), galactanase, xylanase, followed by protease for 24 hours at 37° C (Rizk *et al.*, 2000). To prevent bacterial contamination during the long enzyme treatments, a thin layer of toluene was added. The samples were next dialysed before ethanol was added to 70% (v/v) final concentration. The xyloglucan was allowed to precipitate overnight and stored in deep-freeze. The xyloglucan was washed with buffer or water before use. Xyloglucan content was determined by the iodine-sodium sulphate method (Kooiman, 1960; Hayashi *et al*, 1980) where 1ml of prepared xyloglucan was added to 0.5ml of 0.5% iodine solution (in 1% KI) and 5ml of 20% sodium sulphate solution. Absorbance was read at 640nm after leaving the samples 1hour in the dark at 25°C.

M. Binding assays

The prepared cell wall pellets or the prepared pea xyloglucan (1mg/incubation) were resuspended in 0.3ml incubation buffer (10mmol.l^{-1} oxalic acid/10mmol.l^{-1} sodium

phosphate buffer, adjusted to the correct pH with HCl or NaOH) and incubated with 0.3ml of the [^{14}C]-labelled material at 25°C for 5 minutes. [^{14}C]-galactan or UDP-[^{14}C]-glucuronic acid labelled pectin (20-30 Bq) or the products of these polysaccharides after treatment with enzymes constituted the [^{14}C]-labelled material. The incubation was terminated by centrifuging for 5 minutes at 10000*g*. The pellets were washed once with buffer of the appropriate pH (0.5 ml), centrifuged for 5 minutes, resuspended in 0.4 ml water and mixed with 4ml of scintillation fluid to assay for radioactivity.

N. Exogenous pea pectin or pea polysaccharide preparation

Exogenous pea pectin was prepared by extracting cell wall pellets (see section K, for preparation) with 50mmol.l^{-1} EDTA-phosphate buffer (pH 6.8) for 10 minutes at 100°C. The extract was next desalted by passing it through a Bio-Gel P10 column. The high molecular weight material was collected and concentrated to form the exogenous pea pectin.

The pea polysaccharide was prepared through the same procedure but by extracting the cell wall pellets with 4% KOH (in the presence of 0.1% $NaBH_4$) after the EDTA/phosphate extraction. The alkali extraction is next neutralised and dialysed before being used.

O. Test for β-galactosidase activity

The activity of β-galactosidase was assayed according to the method of Fry (1988). 0.5ml of 5mmol.l^{-1} para-nitrophenyl-β-D-galactoside (ρNP-galactoside) in buffer (50mmol.l^{-1} acetic acid, pH 4.7) was incubated with tested enzyme (0.5ml) for certain

time intervals. The incubations were terminated with 1mol.l^{-1} sodium carbonate. The solution was then assayed spectrophotometrically at wavelength 400nm to check for the ρNP that results from the hydrolysis of ρNP-galactoside and that has a yellow colour in alkaline medium.

P. Viscometric analysis

Viscometric analysis was performed in a Cannon-Fenske Routine Viscometer (Industrial Research Glassware, Ltd., Roselle, New Jersey 07203) to study the rate of flow of 1% and 0.5% pectin or xyloglucan with our without enzymes. The analysis was carried out at 40°C or according to the assay conditions.

Q. Radioactivity Determination

Radioactivity was determined using an LKB 1217 liquid scintillation counter. Samples were assayed for radioactivity for 5 minutes in a biodegradable scintillation fluid known as Ecosint.

Where indicated results are expressed as the mean +/- the standard deviation between the experimental values (2 replicates). The results were repeated at least twice from different particulate enzyme preparations and representative data is shown.

Results

Chapter III

Optimisation of Galactan Synthase Assay

Chapter III

Introduction

Particulate membrane preparations prepared from etiolated pea epicotyls were assayed for galactosyltransferase activity using UDP-[U-^{14}C]-galactose. The total incorporation into ethanol-insoluble material cannot be taken solely as a measure of galactan synthesis, since the galactosyltransferases present in these incubations may transfer the radioactive label into any of at least three products: galactan, xyloglucan, and glycoprotein (Dani, 1996; Baydoun *et. al*, 2001). The aim of the work presented in this chapter was to optimise the conditions of the enzyme galactan synthase involved in the production of β-(1,4)-galactan. Thus optimal conditions were chosen according to digestibility of product by endo-1,4-β-galactanase. Another approach was to study different extraction or solubilisation methods to help in the analysis of the galactan product.

A. Determination of optimum conditions for galactan synthase assay

1-Cation Effect

The effect of adding different divalent cations or EDTA (a chelating agent) to the particulate enzyme preparation was investigated. Compared to the control (with no addition of cations), $MnCl_2$ [Fig 7] was found to give the best incorporation of radioactivity from UDP-[U-^{14}C]-galactose into the galactan product (radioactive material solubilised by endo-1,4-β-galactanase). $CoCl_2$, $CaCl_2$, $MgCl_2$, $NiCl_2$ had a less

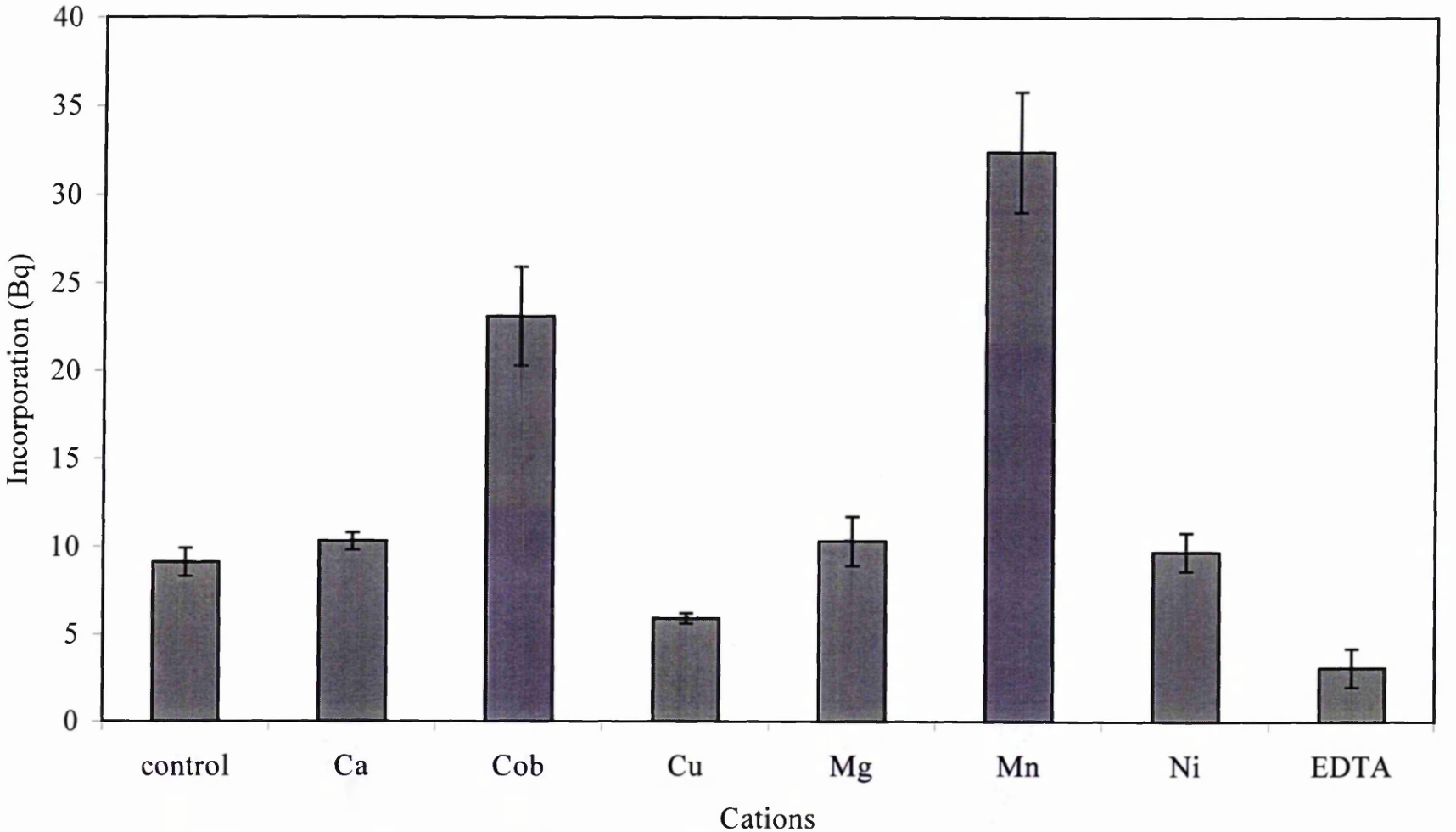

Fig 7. Effect of divalent cations and EDTA on incorporation of radioactivity from UDP-[U-^{14}C]-galactose into galactan product. Standard incubations were carried out, except the following additions [at 10mmol.l^{-1} in each case] were made in place of $MnCl_2$. The particulate product was digested with endo-1,4-β-galactanase to determine the galactan product.

stimulatory effect. $CuCl_2$ and EDTA had an inhibitory effect. When the effect of different concentrations of $MnCl_2$ was investigated, it was found that 10mmol.l^{-1} $MnCl_2$ resulted in the best incorporation [Fig 8].

2-Time-course of incorporation of radioactivity

Incorporation of radioactive galactose into galactan was found to be time-dependent [Fig 9]. The non-linearity of the time-course prevented the accurate determination of kinetic characteristics. This non-linearity may be partly due to an endogenous galactanase present in the membrane preparations as shown in later experiments. Based on this result, all further incubations were carried out for 60 minutes.

3-Effect of adding non-radioactive sugar nucleotides

The effect of adding uridine-diphospho-D-xylose (UDP-xylose) and uridine-diphospho-D-glucose (UDP-glucose) both separately and together to the standard incubation was studied to be able to compare with galactosyltransferase enzyme investigated in peas by Dani (1996). These non-radioactive sugar nucleotides were expected to stimulate incorporation of UDP-[U-^{14}C]-galactose into xyloglucan since both xylose and glucose are part of the main xyloglucan heptasaccharide unit. But as in the work of Dani (1996), the presence of these sugar nucleotides inhibited the incorporation of UDP-[U-^{14}C]-galactose into the particulate enzyme preparation in an additive manner, producing together almost a 60% inhibition [Table 1]. The inhibiting effect was much less evident for galactan synthesis [Table 1].

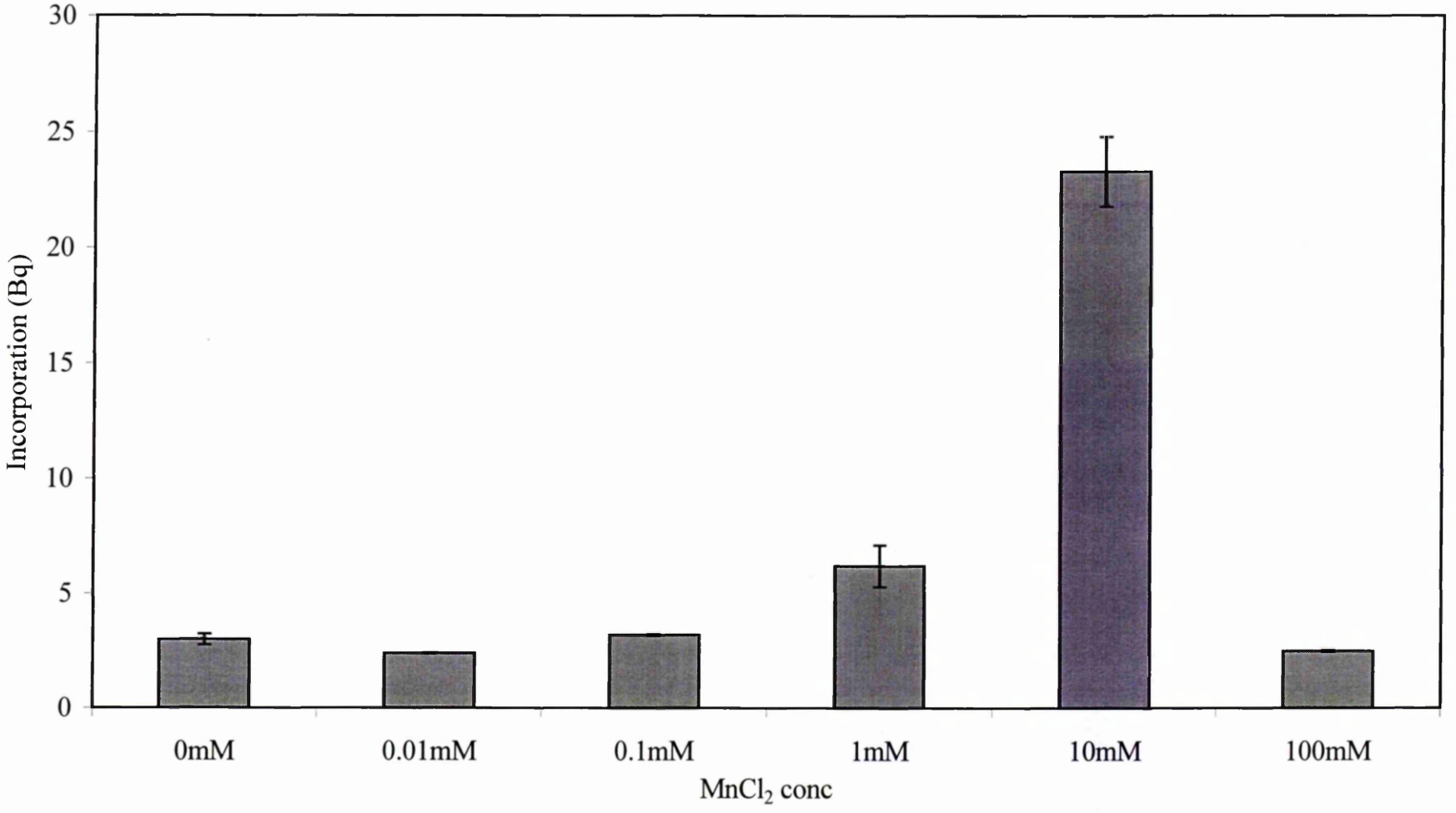

Fig 8. Effect of different concentrations of $MnCl_2$ on incorporation of radioactivity from UDP-[U-^{14}C] - galactose into galactan product. Standard incubations were carried out, except that the concentration of $MnCl_2$ was varied. The control (0mM) had water instead of the divalent cation. The particulate product was digested with endo-1,4-β-galactanase to determine the galactan product.

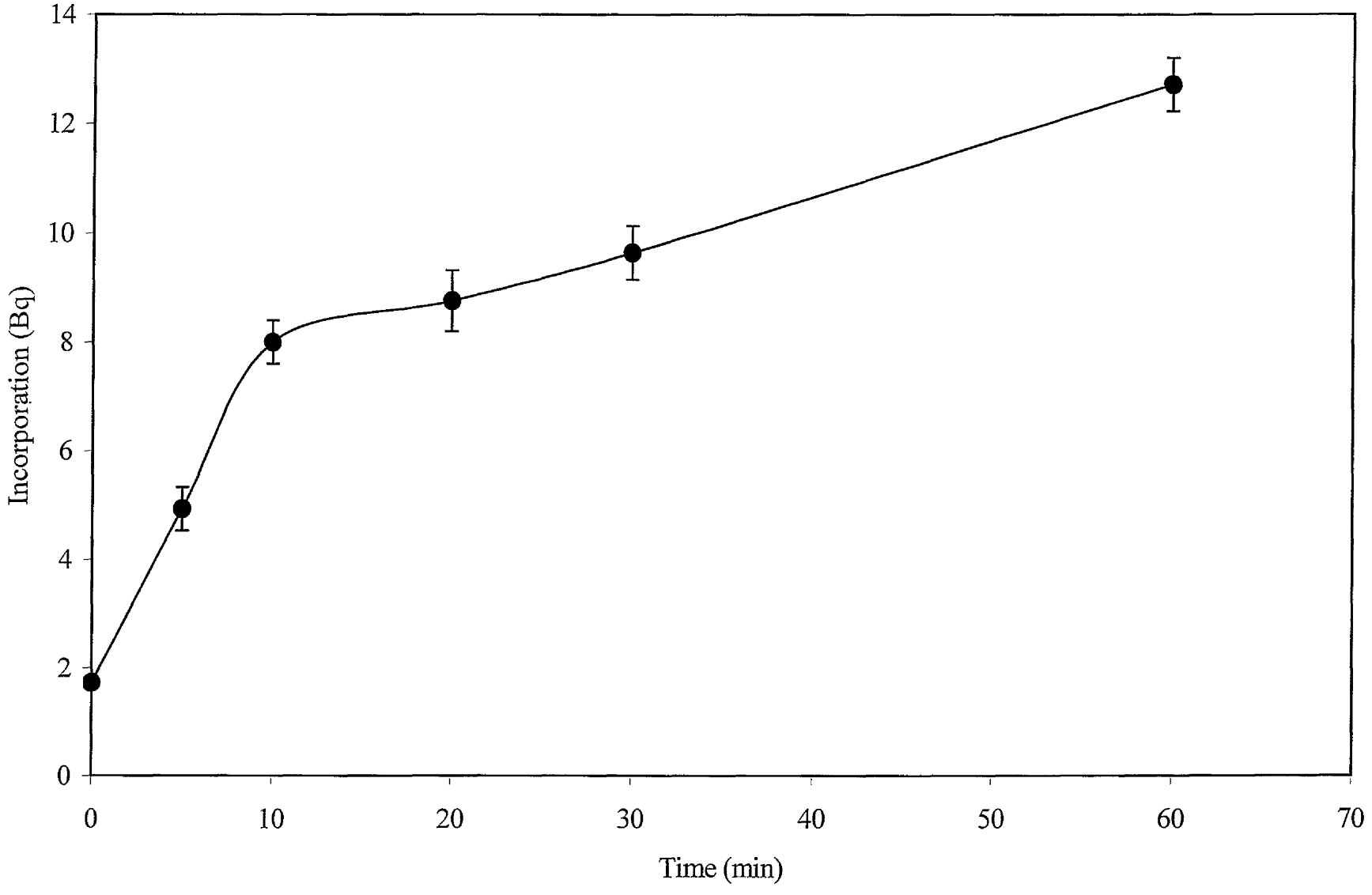

Fig 9. Time-course of incorporation of UDP-[U-^{14}C]-galactose into galactan product. Standard incubations were used, except that the time of incorporation was varied. The particulate product was digested with endo-1,4-β-galactanase to determine the galactan product.

Table 1. Effect of adding UDP-xylose and UDP-glucose on the incorporation of radioactivity from UDP-D-[U-^{14}C]-galactose into the particulate enzyme preparation (total) and into galactan product (SN). Standard incubations were used except that the sugar nucleotides were either present or absent at their respective concentrations as shown. Products were treated with galactanase and radioactivity in the supernatant (SN) and pellet was determined.

Sugar	Incorporation of radioactivity into particulate enzyme preparation (total) (Bq)	% Inhibition (total)	Incorporation of radioactivity into galactan product (Bq)	% Galactan
None	93.00 ± 3.00	0%	57.69 ± 0.55	62%
UDP-xylose (20μmol.l^{-1})	65.11 ± 2.80	30%	46.42 ± 1.00	71%
UDP-glucose (2mmol.l^{-1})	62.13 ± 2.15	33%	52.53 ± 1.00	84%
Both	38.04 ± 0.76	59%	30.33 ± 0.46	80%

4-pH effect

The optimum pH for the incorporation of radioactivity into the particulate enzyme preparation was studied over a range of pH 4.0-8.0 with a 50mmol.l^{-1} Mes/Tris HCl buffer. As shown in Fig 10, there exists a broad peak for pH optimum between pH 5 to 7 for the galactan product. Another peak also exists around pH 8. The next chapter shows more detailed work done to optimise the effect of pH.

5-Effect of adding external acceptors

An extensive study was performed to compare the effect of adding galactan, pectin, and RG I on the incorporation of radioactivity [Table 2]. Galactan stimulated the incorporation of UDP-[U-^{14}C]- galactose into particulate enzyme preparation as well as into the galactan product at most concentrations studied. Pectin had little effect while RGI was inhibitory. Triton X-100, when used alone or in the presence of the above external acceptors resulted mainly in a stimulatory effect [Table 3].

6-Effect of varying concentrations of UDP-[U-^{14}C]- galactose

The amount of UDP-[U-^{14}C]- galactose needed to obtain optimal incorporation was about 1.5 KBq (0.0375μCi) resulting in about 6% incorporation into particulate enzyme preparation and 4% incorporation into galactan product [Table 4]. Since this concentration gave the best incorporation, the same concentration was retained when the assay was scaled up to obtain more radioactive product.

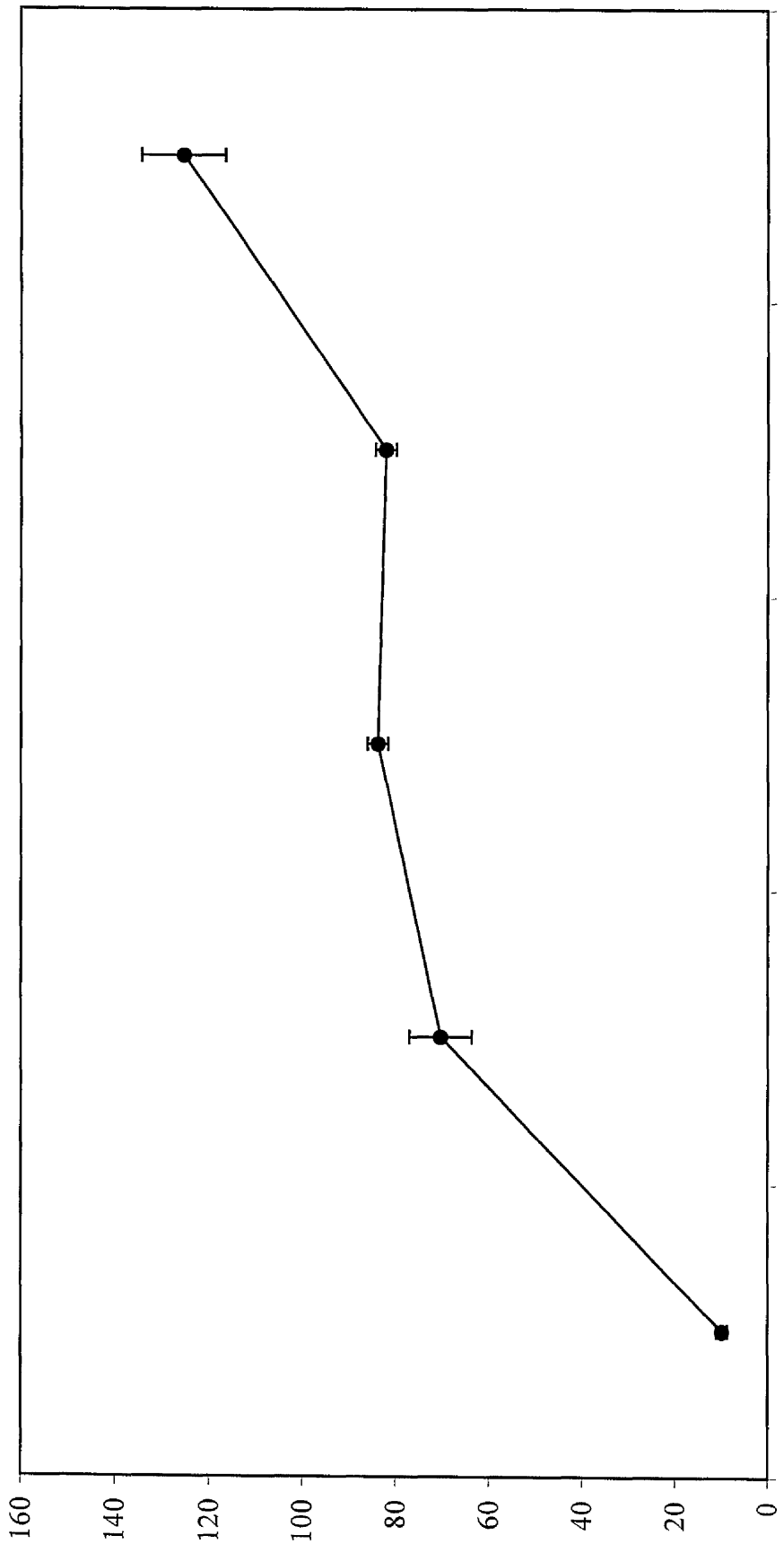

Fig 10. Effect of different pH on incorporation of radioactive galactose into galactan product. Standard incubations were carried out except that different resuspension buffers at the specified pH(s) were used. The particulate product was digested with endo-1,4-β-galactanase to determine the galactan product.

Table 2. Effect of adding external acceptors on the incorporation of radioactivity from UDP-^{14}C-galactose into particulate enzyme preparation (total) and into galactan product (SN). Products were treated with galactanase and radioactivity in the supernatant (SN) and pellet was determined.

Addition	Galactan (Bq)	% Galactan (SN)	Incorporation (total) Bq	% Incorporation into particulate enzyme preparation (total)
None	8.76±0.84	100%	22.20±0.87	100%
Pectin	8.20±1.00	94	22.89±0.90	103
RG I	6.20±0.24	71	20.33±0.84	92
0.05% Galactan	10.98±0.20	125	25.67±0.99	115
0.1% Galactan	9.53±1.00	108	23.96±0.53	107
0.2% Galactan	10.27±.22	117	23.71±0.99	107
0.5% Galactan	11.76±0.60	134	25.28±0.36	114
1% Galactan	10.58±1.01	121	25.33±1.00	114
1% Triton X-100	9.58±0.58	109	25.87±0.80	116

Table 3. Effect of adding 1% Triton X-100 and external acceptors on the incorporation of radioactivity from UDP-^{14}C-galactose into particulate enzyme preparation (total) and into galactan product (SN). Products were treated with galactanase and radioactivity in the supernatant (SN) and pellet were determined.

Addition	Galactan (Bq)	% Galactan (SN)	Incorporation (total) Bq	% Incorporation (total)
Triton X-100	9.58±0.58	100%	25.87±0.80	100%
Pectin + Triton X-100	8.04±1.00	84	27.47±0.90	106
RG1+ Triton X-100	6.73±0.98	70	20.09±0.99	78
0.05% Galactan + Triton X-100	12.22±0.97	128	29.98±0.99	116
0.1% Galactan + Triton X-100	11.42±0.11	119	29.96±0.47	116
0.2% Galactan + Triton X-100	17.22±1.00	180	38.62±1.00	149
0.5% Galactan + Triton X-100	12.67±1.00	132	28.51±0.99	110
1% Galactan + Triton X-100	16.69±0.99	174	31.53±1.20	122

Table 4. Effect of varying amount of UDP-^{14}C-galactose on the incorporation of radioactivity into particulate enzyme preparation (total) and into galactan product (SN). Products were treated with galactanase enzyme and radioactivity in the supernatant (SN) and pellet were determined.

Amount of radioactive UDP-^{14}C-galactose and Treatment	Incorporation		% label in SN as compared with total
	% total	% SN	
0.1125nmole, 1467 Bq (control, buffer only)	6.1	3.3	54
0.1125nmole, 1467 Bq (galactanase-treated)	5.8	4.4	76
0.375nmole, 4933Bq (galactanase-treated)	4.9	4.1	83
0.75nmole, 9867Bq (galactanase-treated)	5.6	4.6	82
1.125nmole, 14800Bq (galactanase-treated)	4.4	3.5	80

B. Analysing the product

1-Total acid hydrolysis

Total acid hydrolysis (2mol.l^{-1} TFA at 120°C for 1 hour) of the particulate enzyme preparation, followed by paper chromatography in ethyl acetate/pyridine/water (EPW) 8:2:1 (v/v) revealed that at least 90% of the radioactivity ran as a peak parallel to the marker sugar galactose [Fig 11].

2-Enzyme treatments

To study the solubilisation of the radioactive product due to different enzyme treatments, the washed pellets were treated with different enzymes as described under "Materials and Methods" and the radioactivity was analysed in the supernatant and the pellet [Table 5]. The control included only buffer, as boiling did not always result in complete deactivation of the enzyme. There was around 55% solubilisation of galactan product due to galactanase, 65% due to pectin lyase, 52% due to protease of bacterial origin, and 72% solubilisation due to protease of fungal origin (Proteinase K).

3-Solubilisation of galactan product

In an attempt to solubilise the galactan product that was being synthesised before treating it with enzymes, two approaches were used. The first approach was to use the standard method of terminating the incubation reaction with 90% ethanol, then washing the insoluble material with 70% ethanol (3x) followed by a water wash. The galactan

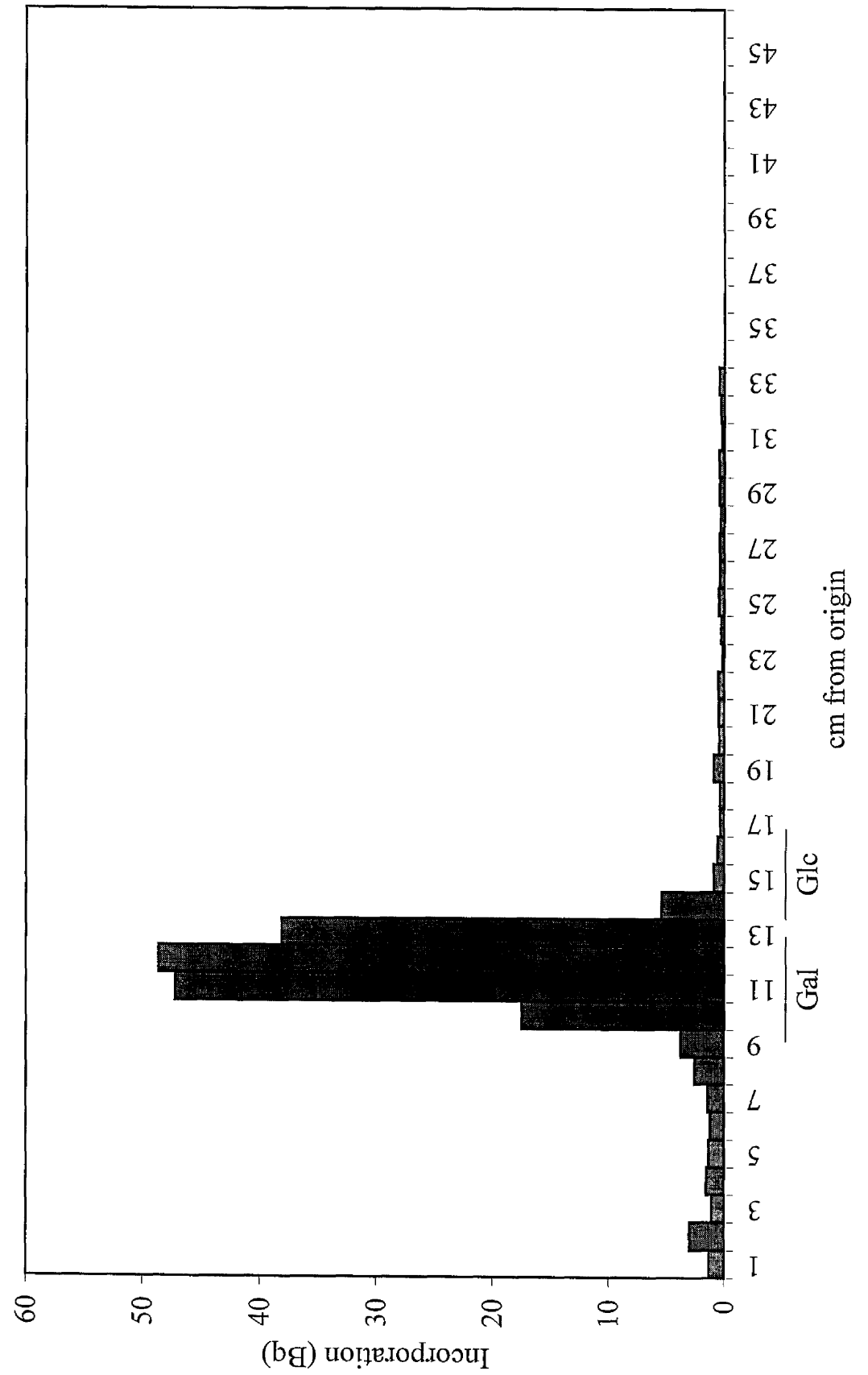

Fig 11. Descending paper chromatography of the complete TFA hydrolysate of the particulate enzyme preparation in 8:2:1 ethyl acetate: pyridine: water (v/v) system for 18 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining.

Table 5. Effect of solubilisation of the radioactive material to the supernatant (SN) by treatment with different enzymes. Standard incubations were used. Controls had buffer only (no enzyme).

Treatment	Solubilisation in SN (%)	Solubilisation in SN minus Control (%)
ctrl (pH4.5 buffer, 25°C)	11%	----------
ctrl (pH4.5 buffer, 40°C)	14%	----------
Galactanase (40°C)	69%	55%
Pectin Lyase (40°C)	79%	65%
Protease (Bacterial, 40°C)	66%	52%
Protease (Fungal, 40°C)	86%	72%

product was then extracted with either 1ml of 50mmol.l^{-1} EDTA-phosphate buffer (pH6.8) or 1ml of water at 100°C for 10 min and the extracts were run on Bio-Gel P10 [Fig 12]. The EDTA-phosphate extract was found to be richer in the high molecular weight material than the water extract.

In the second approach, the incubation reaction was terminated by boiling for 10 minutes instead of adding 90% ethanol and no washes were performed to avoid loosing any galactan product. Thus all the low molecular weight incubation products remained in the subsequent analysis. The pellets were extracted with EDTA-phosphate buffer or water as in the first method. As expected, when run on Bio-Gel P10 the low molecular weight region had very high counts as the pellets contained the unspecifically bound or excess UDP-[U-^{14}C]- galactose [Fig 13]. No problem was expected as the product of interest in this study lies in the high molecular weight region. But after running UDP-[U-^{14}C]-galactose on Bio-Gel P10, it was found that the radioactivity in the high molecular weight region was enough to obscure the radioactivity of the product that is being synthesised [Fig 14]. Therefore, this method had to be abandoned and all future extractions followed only the first approach.

4-Analysis of the water wash

Some investigators reported that galactan was water-soluble [McNab *et al.* (1968) and Goubet and Morvan (1993)]. Thus, to make sure that the product is not being washed out, the water wash was run on Bio-Gel P10 to check if much material is lost in the high molecular weight region [Fig 15]. Only 4.4% of the water wash was of high molecular

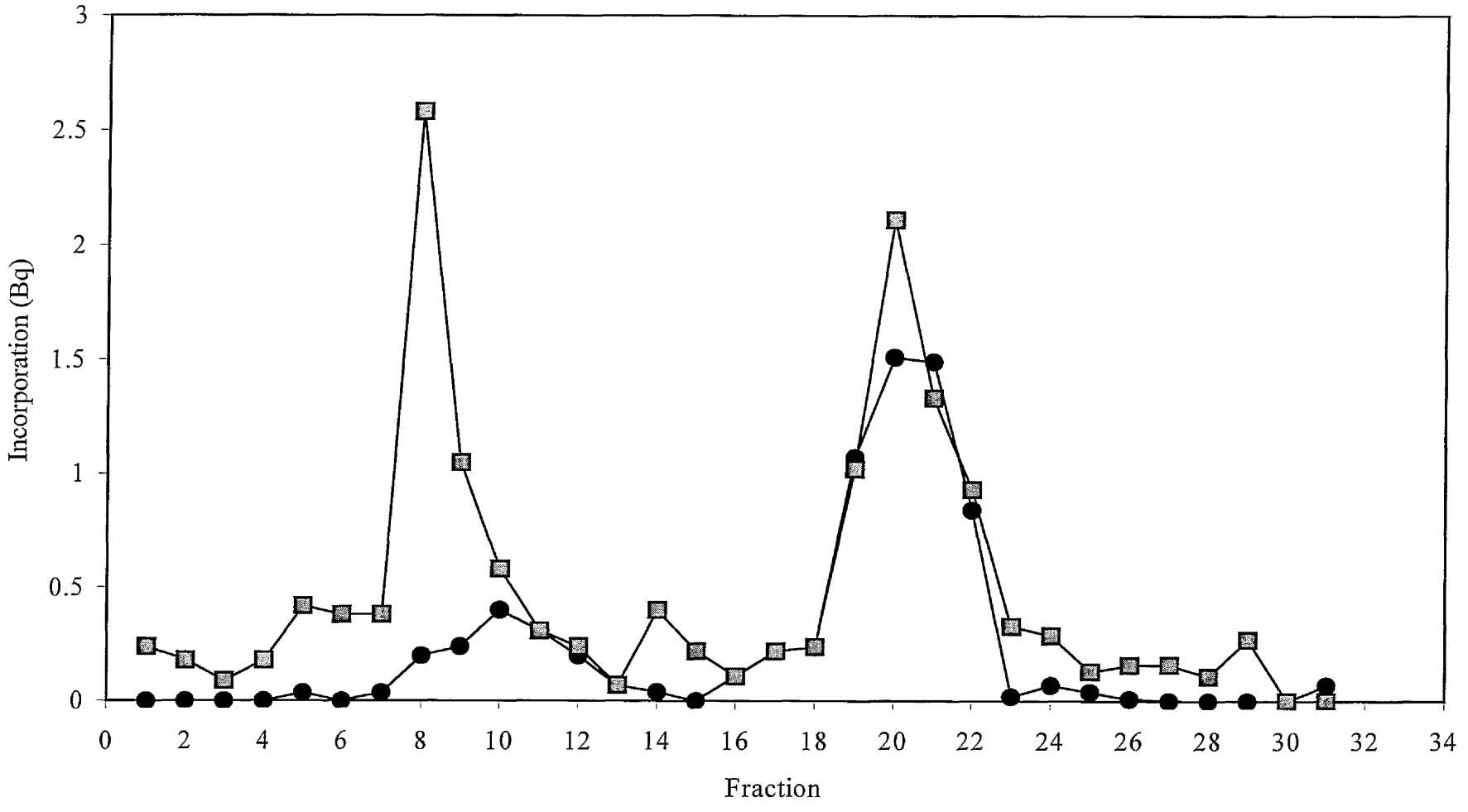

Fig 12. Gel filtration on Bio-Gel P10 column after extracting the standard particulate enzyme preparation with hot EDTA-phosphate or hot water (100°C). Squares represent the material extracted by EDTA-phosphate, while circles represent the material extracted by hot water. The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 18-23.

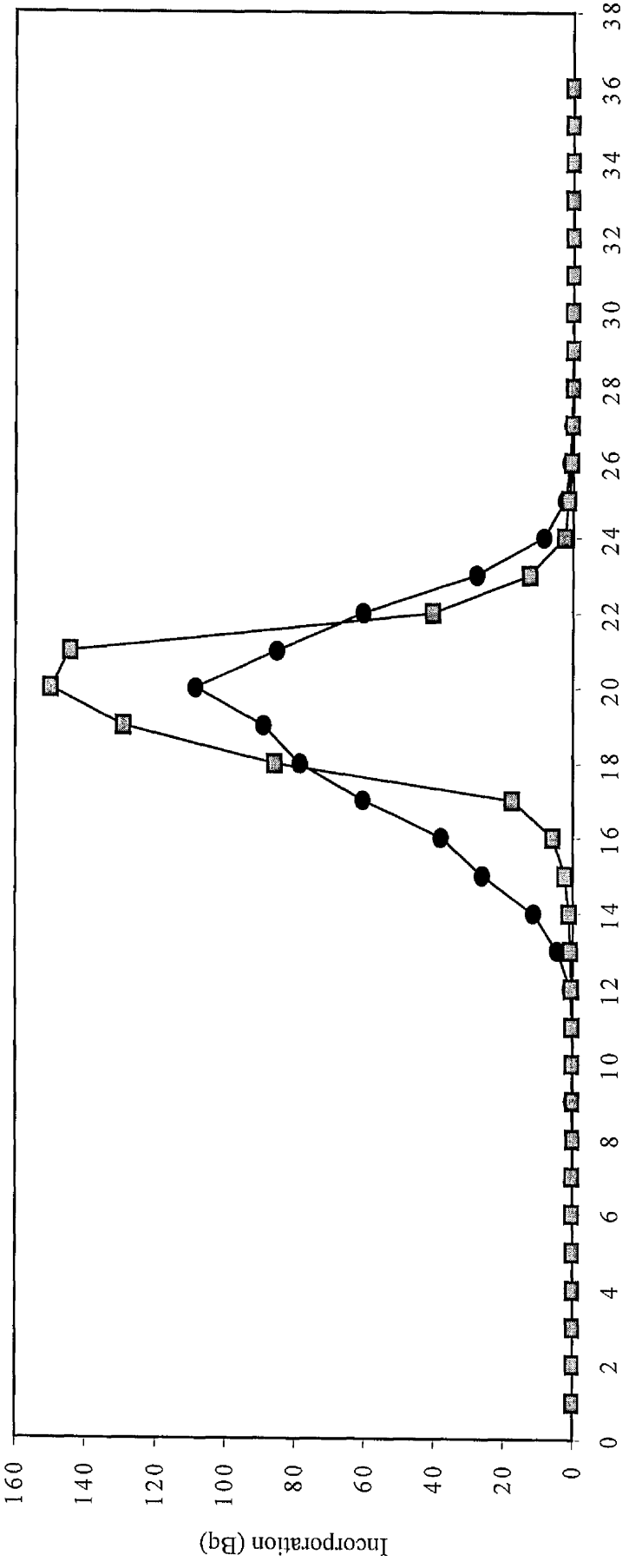

Fig 13. Gel filtration on Bio-Gel P10 column after extracting the unwashed particulate preparation with hot EDTA-phosphate or hot water (100°C). The incubation of the particulate enzyme preparation was not terminated by ethanol (but by boiling) nor was it washed as the standard particulate preparations. Squares represent the material extracted by EDTA-phosphate, while circles represent the material extracted by hot water. The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 18-23.

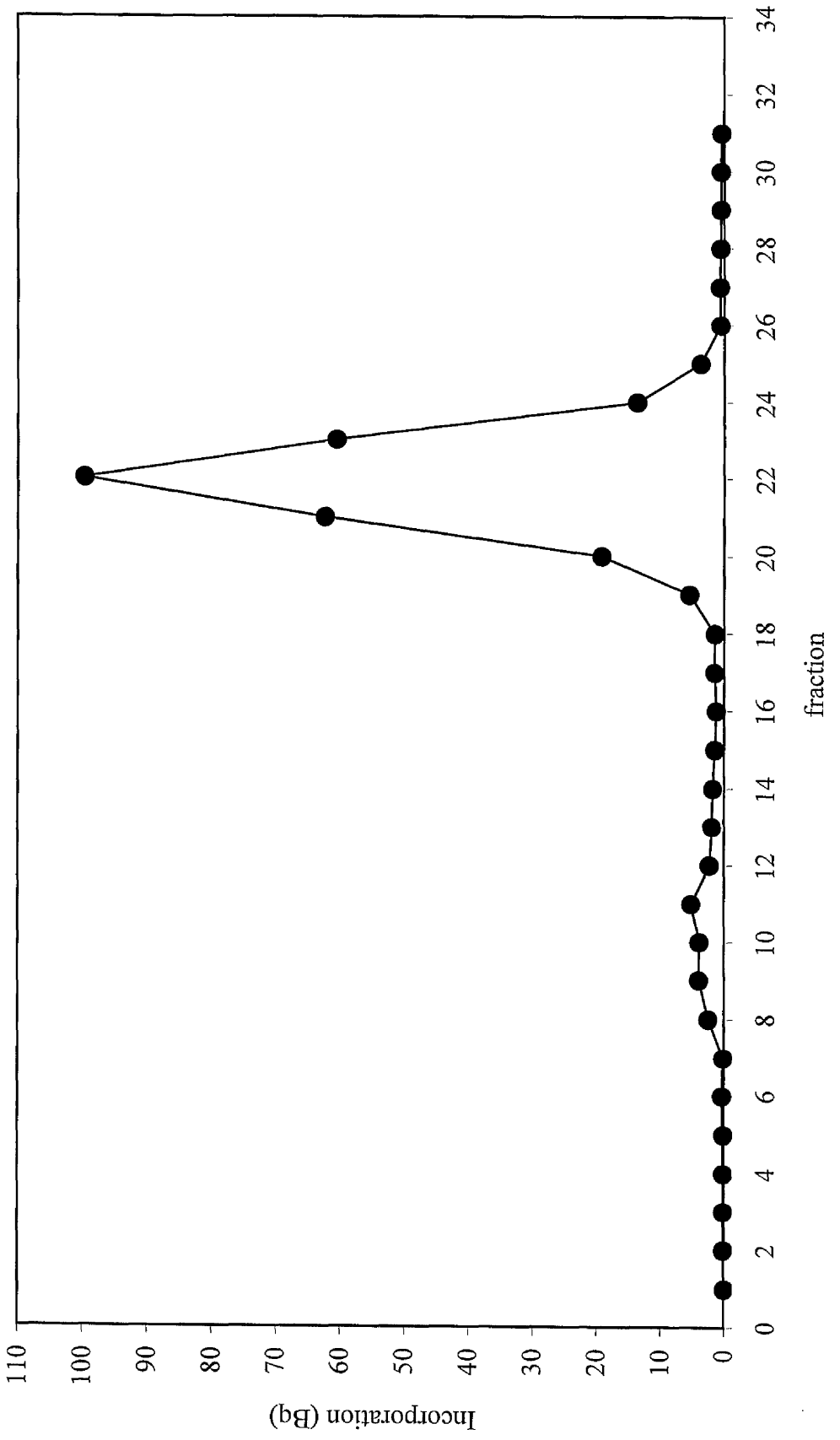

Fig 14. Gel filtration on Bio-Gel P10 column of UDP-[U-^{14}C]-galactose. The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 18-23.

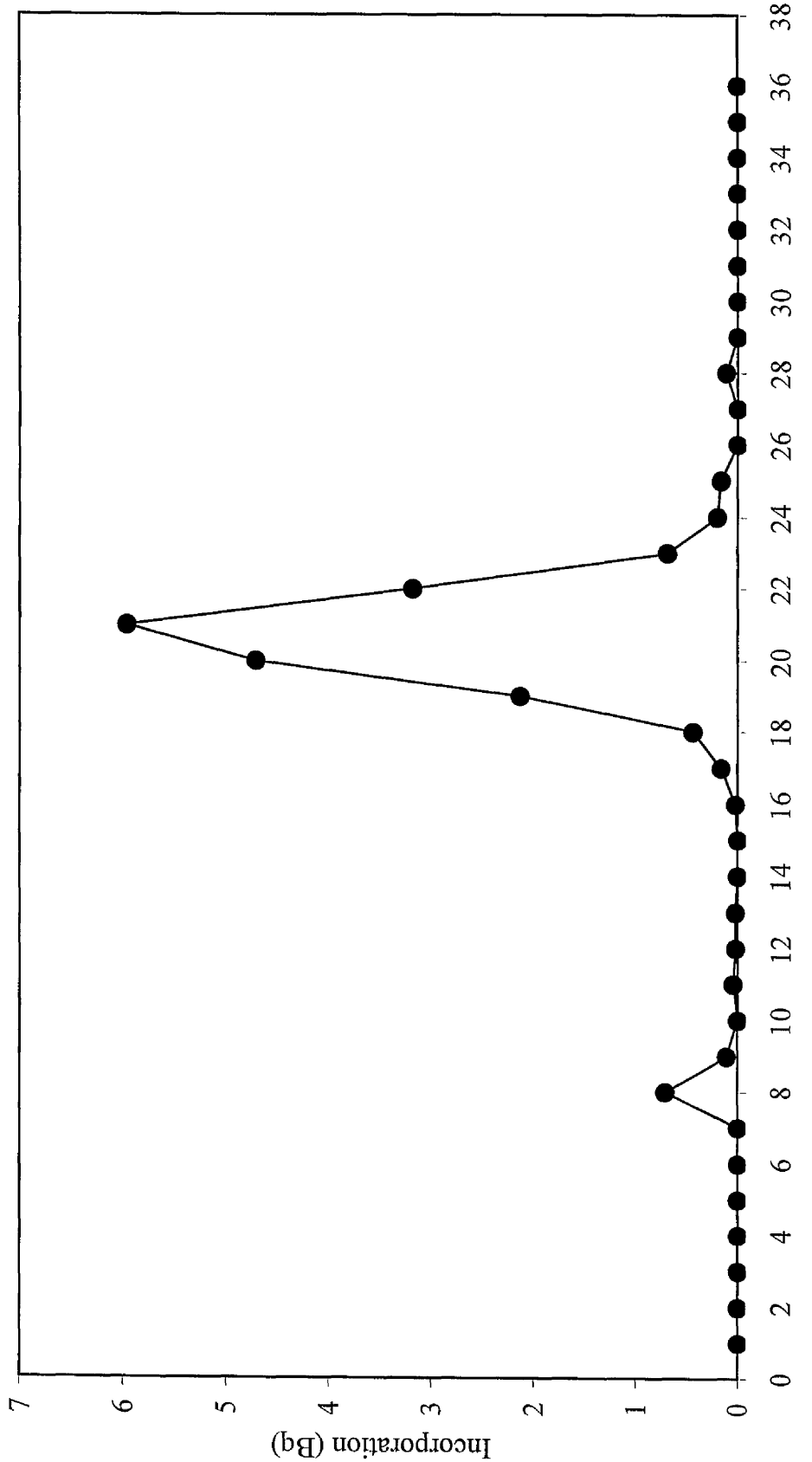

Fig 15. Gel filtration on Bio-Gel P10 column of the standard water wash of the particulate enzyme preparation. The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 18-23.

weight material, thus the standard procedure of washing with water after the ethanol washes was continued for the rest of the experiments.

5-Extractions and enzyme treatments

To be able to further characterise the product, different extractions were performed. The particulate enzyme preparation was treated with EDTA-phosphate buffer ($50mmol.l^{-1}$, pH6.8), water, 4%KOH (containing 0.1%$NaBH_4$) at different temperatures [Table 6]. It was found that hot EDTA-phosphate buffer and KOH gave the best solubilisation, thus pellets were extracted with them consecutively. These extractions were then concentrated and the KOH extract was neutralised and both were run on Bio-Gel P10 column [Fig 16]. Much of the high molecular weight material was solubilised by the first EDTA-phosphate buffer while some remained insoluble with EDTA-phosphate buffer but soluble with KOH.

Since the product of interest in this study lies in the high molecular weight region, the high molecular weight fractions from the extracts were treated with galactanase or other enzymes, then run on a Bio-Gel P10 column to compare the changes in molecular weight. It was shown that the hot EDTA-phosphate buffer extract included a galactan [Fig 17]. Galactanase enzyme resulted in approximately 23%, pectin lyase 31%, cellulase 25%, and proteinase K 30% breakdown of the high molecular weight material into intermediate or low molecular weight products. The KOH extract contained a galactan as well as other products as evident in Fig 18.

Table 6. Effect of different extractions on the solubilisation of radioactive material to the supernatant (SN). Standard incubations were used.

Treatments	%SN
Hot water (1ml,100°C,10min)	25%
Water (1ml,25°C,1hr)	12%
Hot EDTA-phosphate buffer (1ml,100°C,10min)	34%
EDTA-phosphate buffer (1ml,25°C,1hr)	14%
4%KOH, 0.1%NaBH4 (1ml,25°C,1hr)	34%

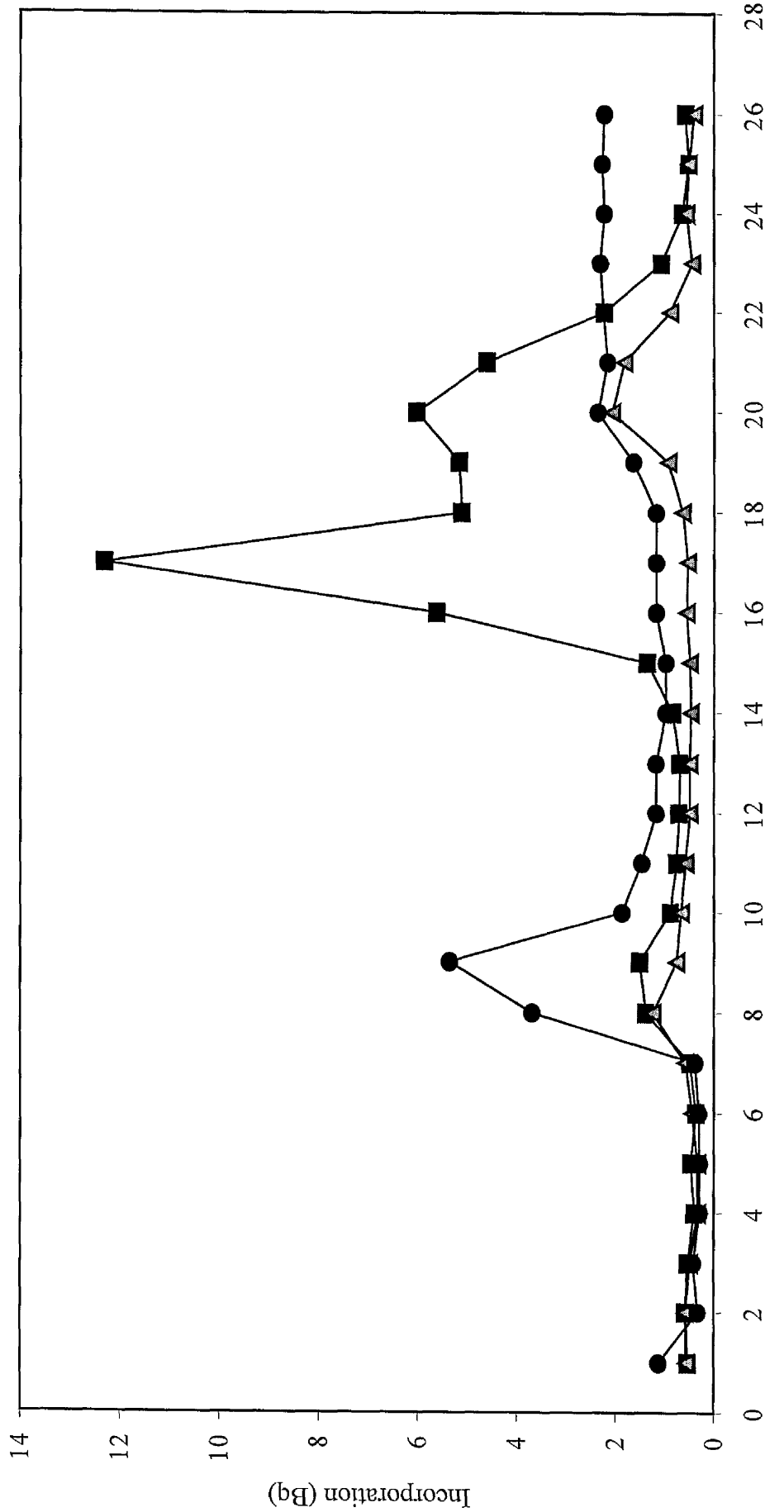

Fig 16. Gel filtration on Bio-Gel P10 column after extracting the particulate enzyme preparation consecutively with hot EDTA-phosphate (twice) followed by 4% KOH/0.1% NaBH4. Squares represent the first EDTA-phosphate extract, triangles represent the second EDTA-phosphate extract, while circles represent the material extracted by 4%KOH. The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 18-23.

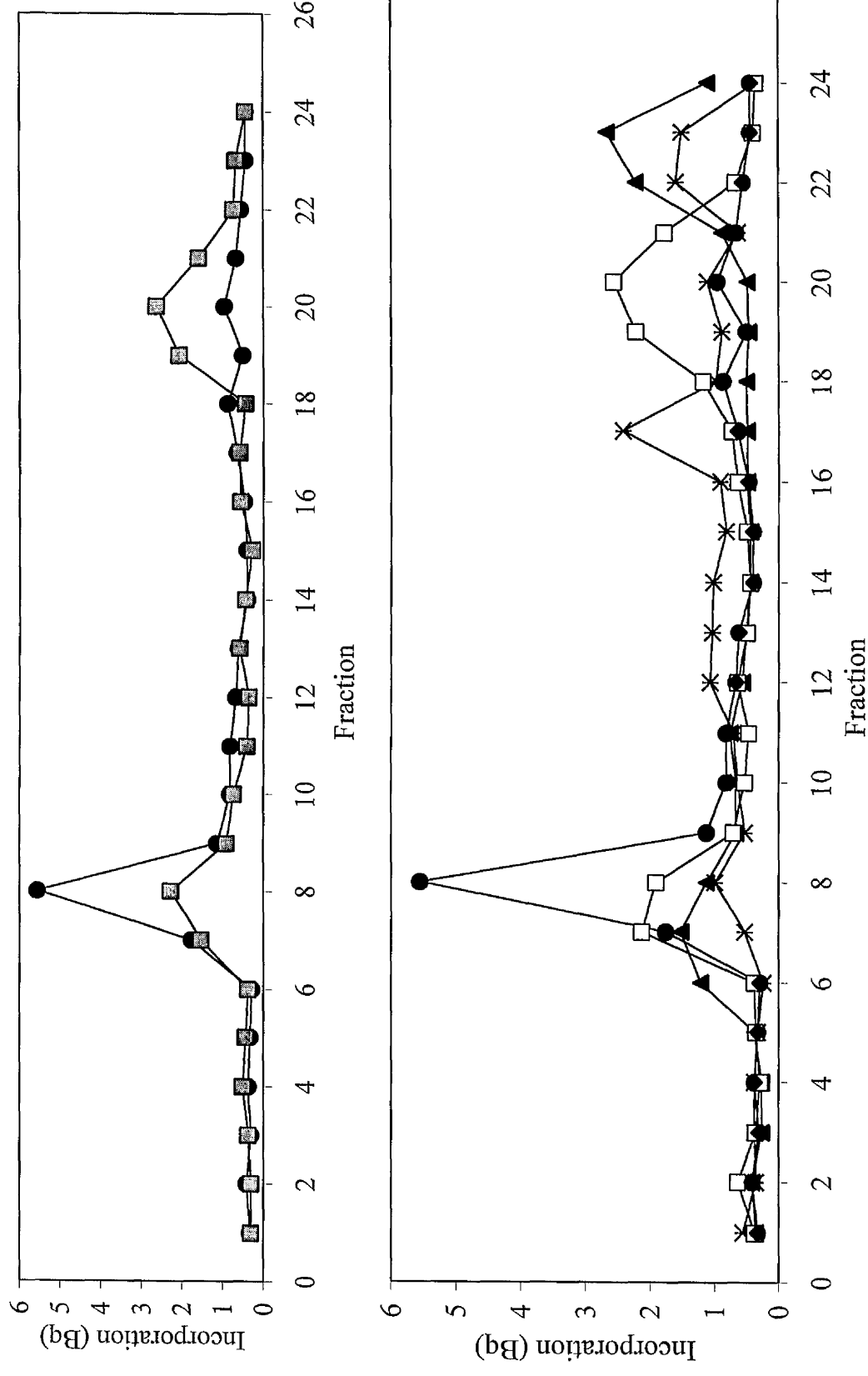

Fig 17. Gel filtration on Bio-Gel P10 column after treating the high molecular weight EDTA extracts of the particulate product with different enzymes. The control (circles) consists of untreated extract only. The extracts were treated with galactanase (closed squares), cellulase (open squares), proteinase K (triangles), or with pectin lyase (crosses). The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 18-23.

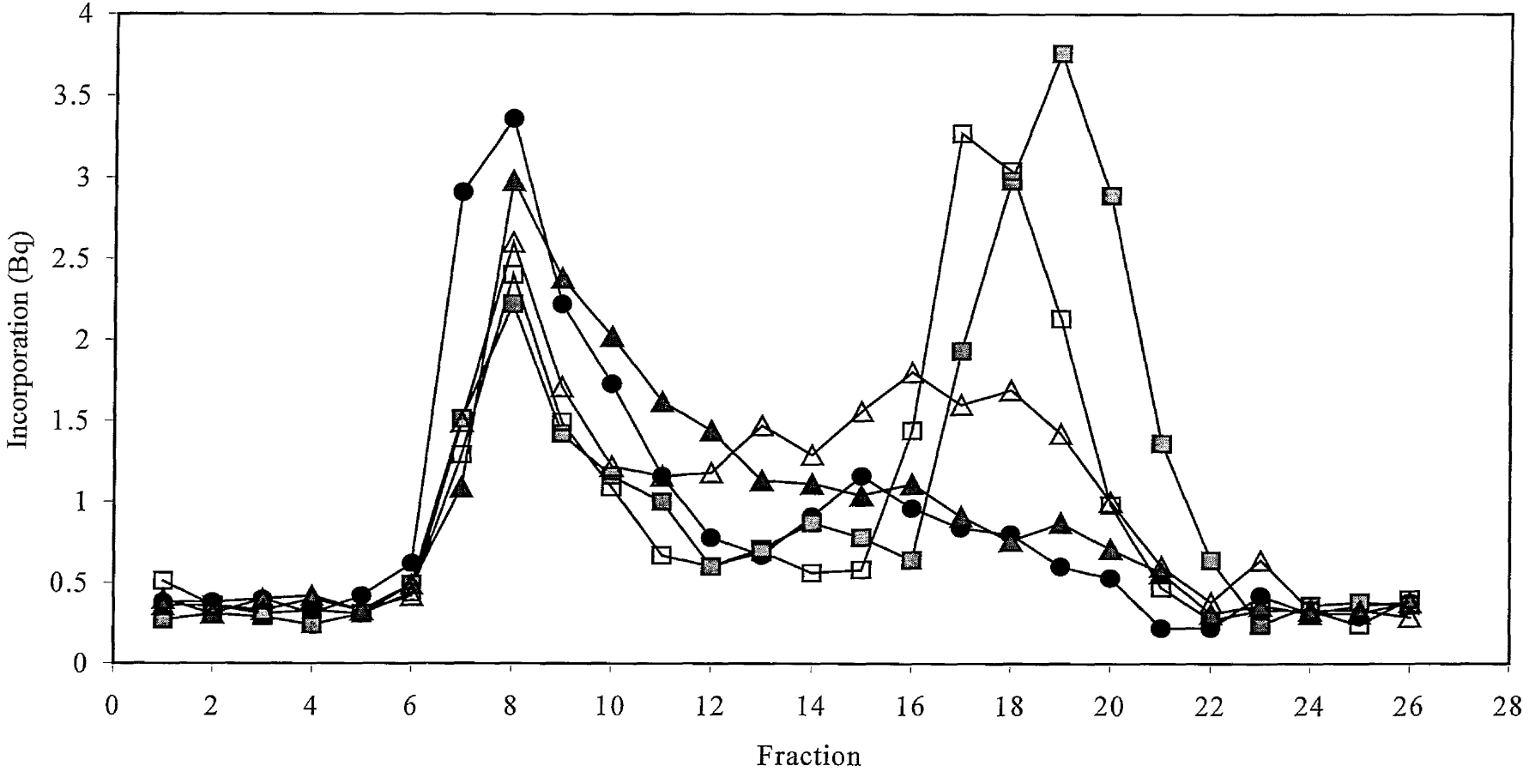

Fig 18. Gel filtration on Bio-Gel P10 column after treating the high molecular weight KOH extracts of the particulate product with different enzymes. The control (circles) consists of untreated extract only. The extracts were treated with galactanase (closed squares), cellulase (open squares), pectin lyase (closed triangles), or with polygalacturonase (open triangles). The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 17-23.

Conclusion

The conditions for galactan synthase in peas (*Pisum Sativum* L. cv Meteor) were partially optimised. 10mmol.l^{-1} $MnCl_2$ resulted in the best incorporation of UDP-[U-^{14}C]-galactose into particulate enzyme preparation. The optimal concentration of radioactive UDP-[U-^{14}C]- galactose and the termination method of the incubation reaction have been established. The results stated in this chapter showed that a galactan was being formed, but further work was still needed to identify the products further and the nature of any polymer to which the galactan was attached. This will be discussed in the next chapters in addition to further optimisation conditions.

Chapter IV

Optimising Conditions Using a Different Preparation Method

Chapter IV

Introduction

In this chapter, further steps were taken towards optimisation since clear-cut optimal conditions with regards to pH and time-course were not yet obtained. The centrifugation protocols and the effect of adding external acceptors were investigated further in order to obtain an increase in the amount of material solubilised by galactanase enzyme and a decrease in the amount of material solubilised in the control.

A. Optimum conditions for galactan synthase assay using different preparation method

1-Optimisation of the centrifugation speed

Experiments were performed in which the initial 13,000 *g* centrifugation speed was varied over a time period of 0, 15, 30, 60, and 120 minutes. This was performed to favour action of galactan synthase enzyme over other active galactosyltransferases and also because it was suspected that the galactan product being formed was degraded with time due to the action of an endogenous enzyme. The aim was to find conditions where maximum amount of galactan was produced (material solubilised by galactanase enzyme) with minimum amount of solubilisation of radioactivity in the control. Centrifugation speeds of 3000, 13,000, and 27,000 *g* were studied [Table 7, 8, 9]. In each case the supernatant from this initial centrifugation was re-centrifuged at 100,000g, and the resulting pellet used for the particulate enzyme preparation. The highest percentage of galactan in the product was after centrifugation at 27,000 *g* [Table 10]. This was the case whether the % incorporation into galactan was calculated just by

Table 7. A time course at 3,000 *g* centrifugation speed was performed and the amount of incorporation of radioactivity from UDP-[^{14}C]-galactose into particulate enzyme preparation was determined. Products were treated with endo-1,4-β-galactanase, and radioactivity in the supernatant was determined. Control treatments have the appropriate buffer (no enzyme).

Time	Incorporation into particulate preparation (Bq)	% solubilised in SN of ctrl	% solubilised in SN by galactanase
0 min	34.18 ± 1.14	70%	76%
15 min	127.60 ± 5.47	61%	82%
30 min	197.39 ± 4.23	65%	83%
60 min	232.59 ± 6.98	64%	84%
120 min	245.97 ± 10.99	63%	82%

Table 8. A time course at 13,000 *g* centrifugation speed was performed and the amount of incorporation of radioactivity from UDP-[^{14}C]-galactose particulate enzyme preparation was determined. Products were treated with endo-1,4-β-galactanase, and radioactivity in the supernatant was determined. Control treatments have the appropriate buffer (no enzyme).

Time	Incorporation into particulate preparation (Bq)	% solubilised in SN of ctrl	% solubilised in SN by galactanase
0 min	44.4 ± 7.00	73%	84%
15 min	139.24 ± 13.36	71%	84%
30 min	172.14 ± 8.68	66%	85%
60 min	237.47 ± 12.8	71%	72%
120 min	267.58 ± 5.4	70%	72%

Table 9. A time course at 27,000 *g* centrifugation speed was performed and the amount of incorporation of radioactivity from UDP-[^{14}C]-galactose into particulate enzyme preparation was determined. Products were treated with endo-1,4-β-galactanase, and radioactivity in the supernatant was determined. Control treatments have the appropriate buffer (no enzyme).

Time	Incorporation into particulate preparation (Bq)	% solubilised in SN of ctrl	% solubilised in SN by galactanase
0 min	34.67 ± 1.4	81%	86%
15 min	81.94 ± 3.58	67%	87%
30 min	129.31 ± 1.82	65%	90%
60 min	170.76 ± 6.44	65%	90%
120 min	197.00 ± 4.76	59%	89%

Table 10. Effect of different centrifugation speeds on percentage of galactan in the product. A time course at each centrifugation speed was performed and the percentage of galactan in the product was determined. To determine the % galactan, the % radioactivity solubilised by endo-1,4-β-galactanase enzyme was subtracted by the % solubilised by buffer alone in the control.

Time	% Galactan at 3000 *g*	% Galactan at 13,000 *g*	% Galactan at 27,000 *g*
0 min	6%	11%	5%
15 min	21%	13%	20%
30 min	18%	19%	25%
1 hr	20%	1%	25%
2 hrs	19%	2%	30%

accounting for the amount solubilised by galactanase or by accounting for the amount solubilised by galactanase minus the amount solubilised in the controls. Incorporation of radioactive galactose was found to be time-dependent [Fig 19]. Based on this result, all further incubations were carried out for 60 minutes. This was chosen in preference to 120 minutes so that some substrate would still be present to transfer UDP-[U-^{14}C]-galactose to exogenous acceptors.

2- Effect of Cation

The effect of adding different divalent cations or EDTA to the particulate enzyme preparation was investigated. The results were similar to those presented in the previous chapter.

3-Effect of adding non-radioactive UDP-galactose

Adding non-radioactive UDP-galactose to the standard incubation had a stimulatory effect [Table 11]. The best incorporation was at 0.2mmol.l^{-1} UDP-galactose final concentration. This concentration was used in the forthcoming experiments for the assays of galactan synthase activity and preparation of [^{14}C]- galactan for analysis. A time-course repeated with this concentration gave similar results to those reported earlier.

4-Effect of pH

The optimum pH for the incorporation of radioactivity into the particulate enzyme preparation was studied over a range of pH 3.0-10.0 with a buffer of 50mmol.l^{-1} Mes/Tris HCl/$NaHCO_3$. As shown in Fig 20, the optimum pH for the particulate

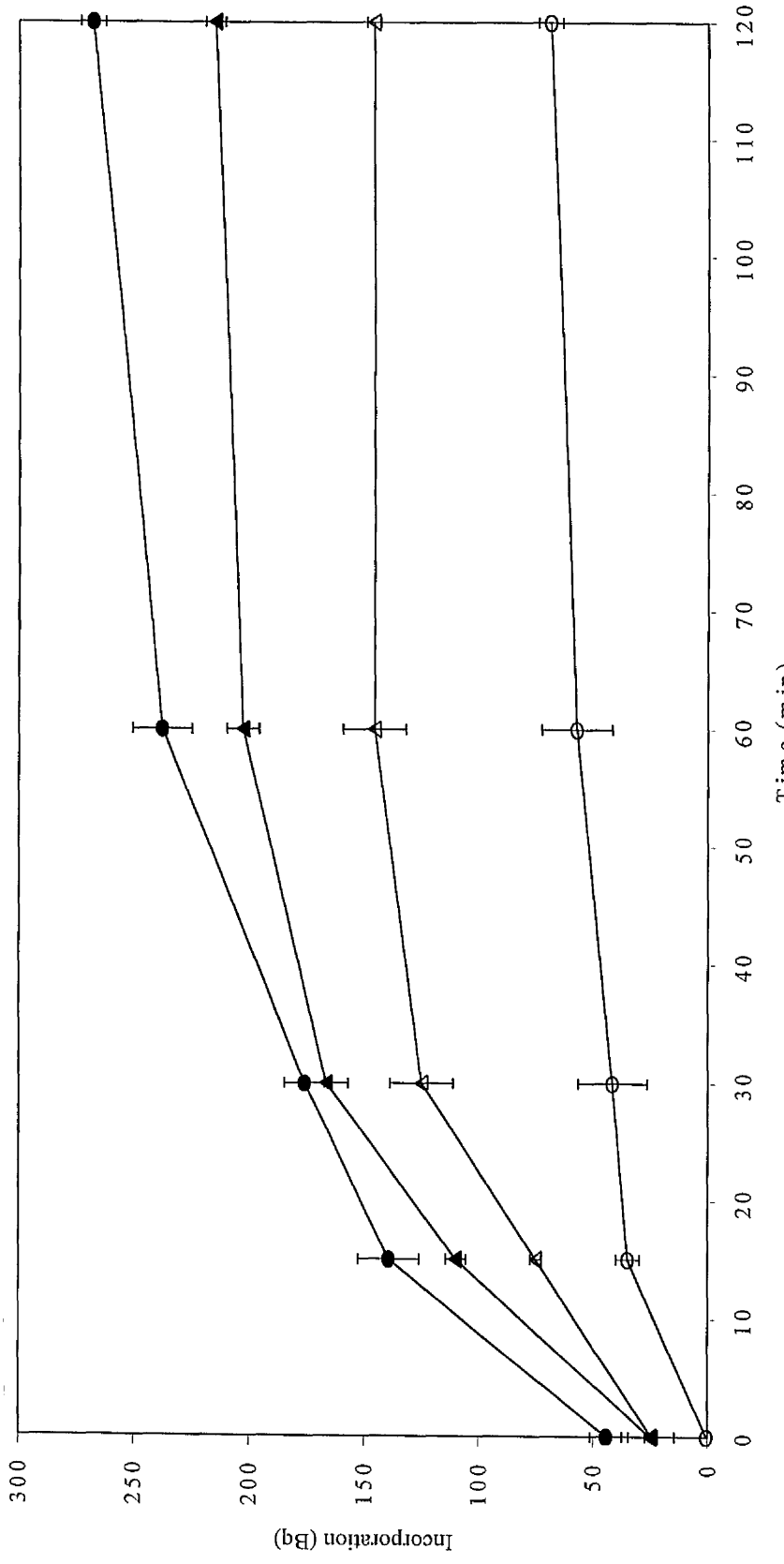

Fig 19. Time-course of incorporation of UDP-[U-^{14}C]-galactose into particulate enzyme preparation (whole pellet) or galactan product. The particulate product was digested with endo-1,4-β-galactanase (in pH 4.5 sodium acetate buffer) to determine the galactan product. Standard incubations were used, except that the time of incorporation was varied. Control treatments were incubated for the same time as treated but no galactanase enzyme was later added (only buffer). Closed circles represent the particulate product, closed triangles represent the galactan product (galactanase solubilised material), open triangles represent the material solubilised in the control treatments, and open circles represent galactanase minus control solubilised material.

Table 11. Effect of adding non-radioactive sugar nucleotide UDP-galactose, of different concentrations, to incubations was investigated. Radioactivity incorporated into particulate preparation or into the galactan product is shown. To determine the % galactan, the % released by endo-1,4-β-galactanase enzyme was subtracted by the % solubilised by buffer alone in the control.

Concentration of UDP-galactose	Radioactivity incorporated into particulate enzyme preparation (Bq x 10^{-2})	% galactan in the particulate enzyme preparation (% solubilised by galactanase - % solubilised by buffer)
2μmol.l^{-1}	1.74 ± 0.01	(79-59) = 20%
10μmol.l^{-1}	1.83 ± 0.10	(81-56) = 25%
20μmol.l^{-1}	1.69 ± 0.12	(82-56) = 26%
40μmol.l^{-1}	1.58 ± 0.22	(81-57) = 24%
0.2mmol.l^{-1}	2.2 ± 0.11	(87-55) = 32%
2mmol.l^{-1}	1.83 ± 0.05	(82-60) = 22%
20mmol.l^{-1}	1.89 ± 0.14	(79-54) = 25%

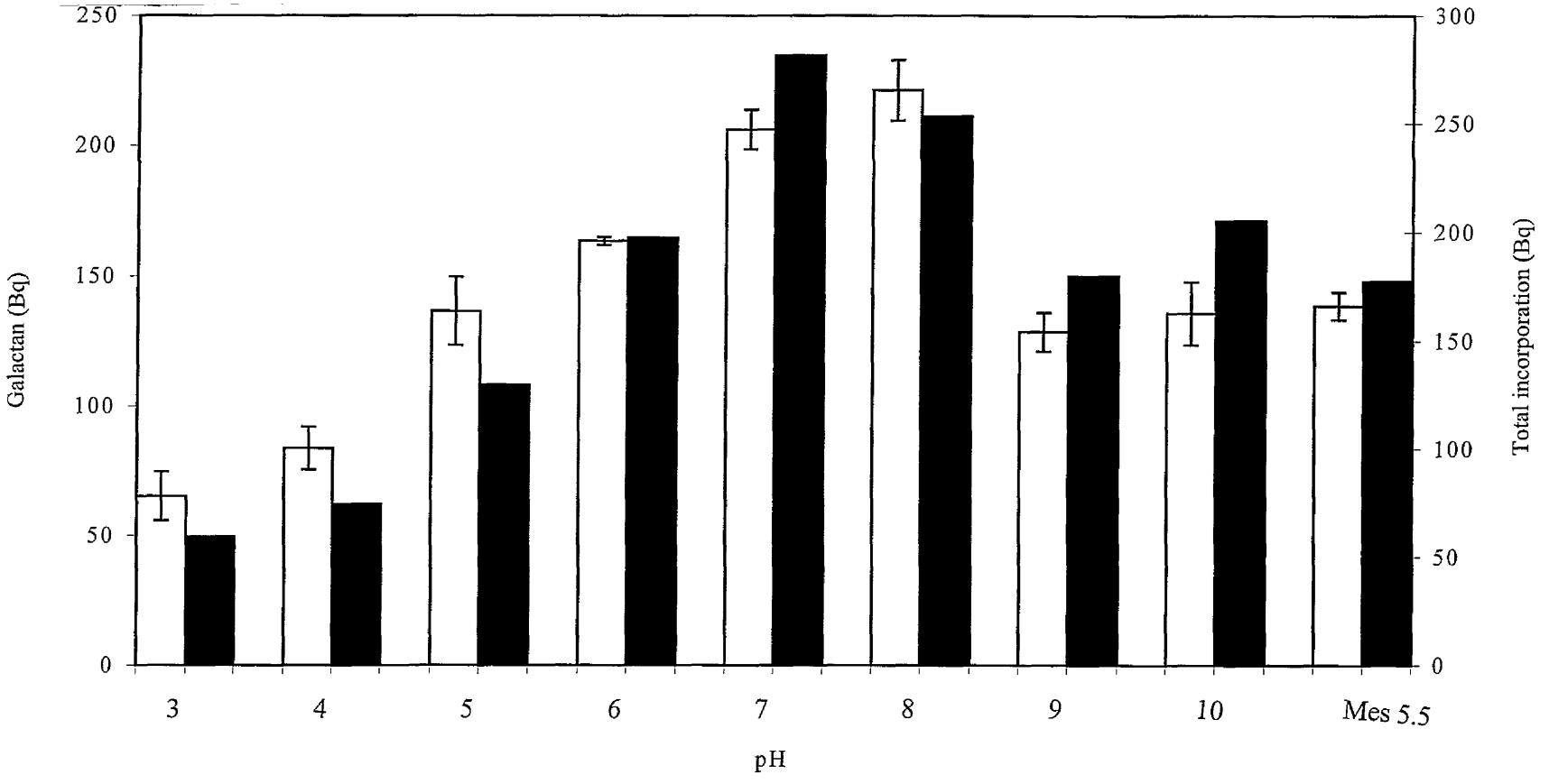

Fig 20. Effect of different pH on incorporation of UDP-[U-^{14}C]-galactose into particulate enzyme preparation (whole pellet) or galactan product. The particulate product was digested with endo-1,4-β-galactanase to determine the galactan product. Standard incubations were used, except that that different resuspension buffers at the specified pH(s) were used. A mixed buffer of 50 mmol.l^{-1} MES/Tris/$NaHCO_3$ was used to vary the pH except at pH 5.5 where MES buffer alone was used (as conditions of all previous experiments). The particulate preparation is denoted by white bars, whereas the galactan product is denoted by black bars. The incorporation into galactan product was not subtracted from control in this experiment.

enzyme preparation was between pH 7 and 8. However, MES buffer (50mmol.l^{-1}) pH 5.5 was found to give the highest percentage of galactan in the product (if accounted for by subtracting the amount solubilised in the controls) as shown by galactanase treatment [Table 12]. EDTA extracts of the incubation products at pH 5.5, 8, and 10 were run on Bio-Gel P10 column [Fig 21]. Both high and low molecular weight products were present at each pH, but the greatest amounts of high molecular weight products were formed at pHs 5.5 and 10. Therefore, the rest of the experiments were carried out at pH 5.5 since pH 10 is not within the normal physiological range. Perhaps, other investigations should consider the nature of the product formed at pH 8.

5-Effect of pH and UDP-galactose concentration

So far, the previous experiments reported in this chapter showed that the optimal UDP-galactose concentration is 0.2mmol.l^{-1} and the optimal pH is 5.5. More experiments were designed to test if a higher concentration of UDP-galactose at pH 5.5 or pH 7.5 may result in:

a) a higher proportion of galactan in the EDTA extract
b) a longer galactan chain that would help in differentiating between action of enzymes.

Incubations of 0.2mmol.l^{-1} or 20mmol.l^{-1} UDP-galactose (x100 or x10,000 of concentration used earlier) at pH 5.5 (MES buffer) and pH 7.5 (MES/Tris/$NaHCO_3$) were assayed. The particulate enzyme preparation was washed as usual and the EDTA extracts were run on Bio-Gel P10 to collect the high molecular weight material. This was divided into three aliquots, and treated with galactanase enzyme, polygalacturonase enzyme, or buffer (control). These treated aliquots were run on Bio-Gel P10 and the

Table 12. Study of the solubilisation of galactan product from particulate enzyme material prepared at different pH. Products were treated with endo-1,4-β-galactanase enzyme and radioactivity in the supernatant was determined.

pH	% radioactivity released by galactanase enzyme
5.5	77%
8.0	74%
10.0	75%

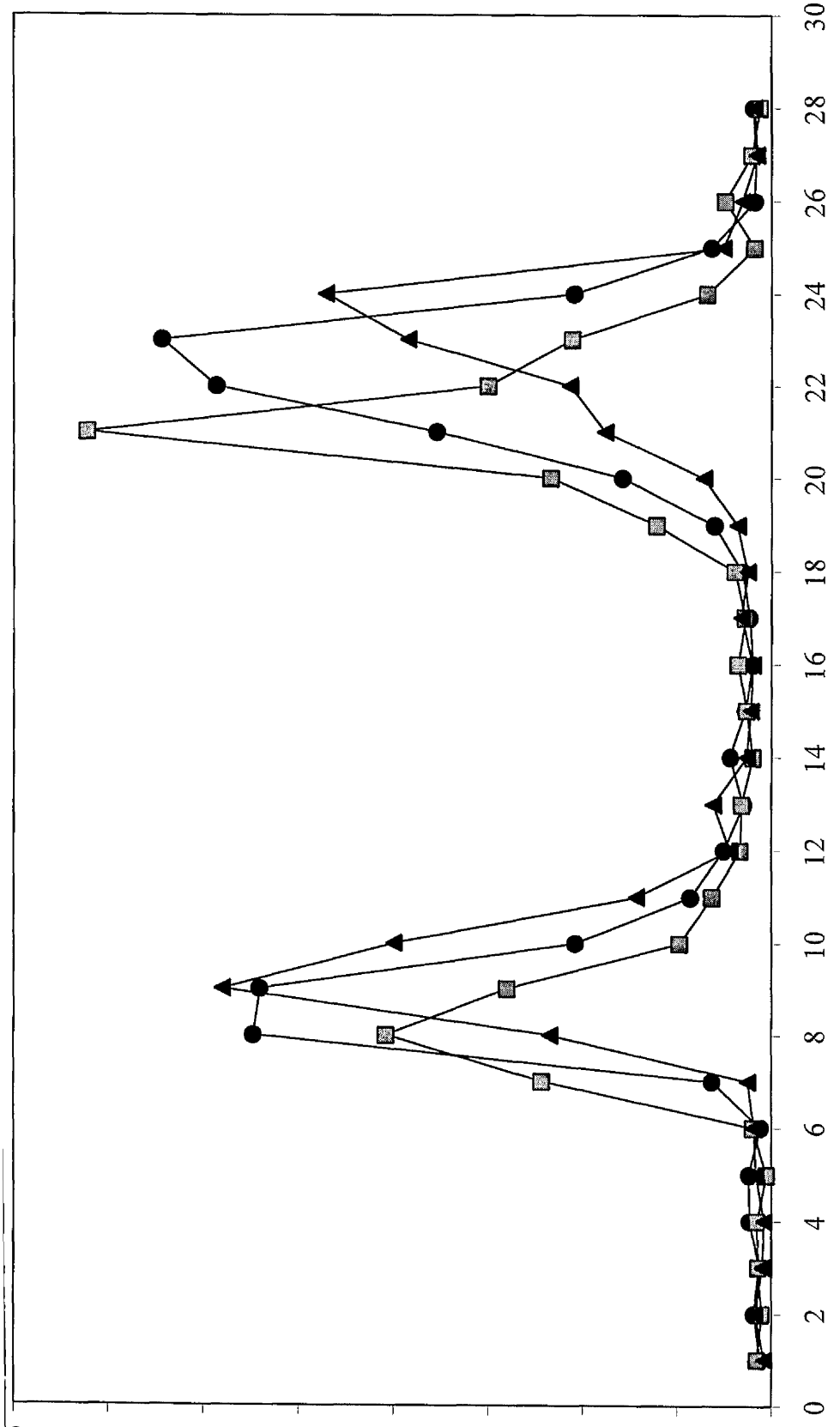

Fig 21. Gel filtration on Bio-Gel P10 column of the EDTA extracts of the particulate enzyme preparations (prepared at different pH). Standard incubations were used, except that different resuspension buffers at the specified pH(s) were used. The Blue Dextran marker ran between fractions 8-10, while the $CoCl_2$ marker ran between fractions 21-24. The EDTA extracts of membranes resuspended at pH 8 (squares), pH5.5 (circles), and pH 10 (triangles) are shown.

collected fractions were assayed for radioactivity [Fig 22]. All the above conditions gave high breakdown with galactanase, but there appeared to be some variation in the degree of breakdown with polygalacturonase enzyme. It may be that different types of pectin (for example: different degrees of methylation or branching) were labelled under different conditions. The more methylated pectin would be more resistant to polygalacturonase breakdown. In any case, pH 5.5 and 0.2mmol.l^{-1} UDP-galactose conditions gave the best representation of high molecular weight EDTA extract broken down by galactanase into low molecular weight product and broken down by polygalacturonase into intermediate and low molecular weight product [Fig 22].

6-Galactanase time-course

The particulate enzyme preparation was subjected to different time intervals of galactanase treatment. After termination, the solubilised supernatant was run on paper chromatography in ethyl acetate/pyridine/water (EPW) 10:4:3 (v/v) for 18 hours [Fig 23]. Pellets were assayed for radioactivity and the % breakdown was calculated [Table 13]. Table 13, shows a large breakdown due to galactanase treatment starting from 15-30 minutes. This breakdown increased gradually with time, but so did the solubilisation due to buffer alone. Hence, it was concluded that it was best not to treat with enzymes overnight (18hours) as done previously. Fig 23, revealed that a large amount of radioactive galactose monosaccharide was present in the pellets as a low molecular weight contaminant. When galactanase is present, the enzyme or the ammonium sulphate in which it is resuspended or both caused this galactose to smear out along the earlier part of the chromatogram, masking any oligosaccharide that might be present as a result of hydrolysis. The peaks in fractions 21-22 and 15-16 suggest the possibility of

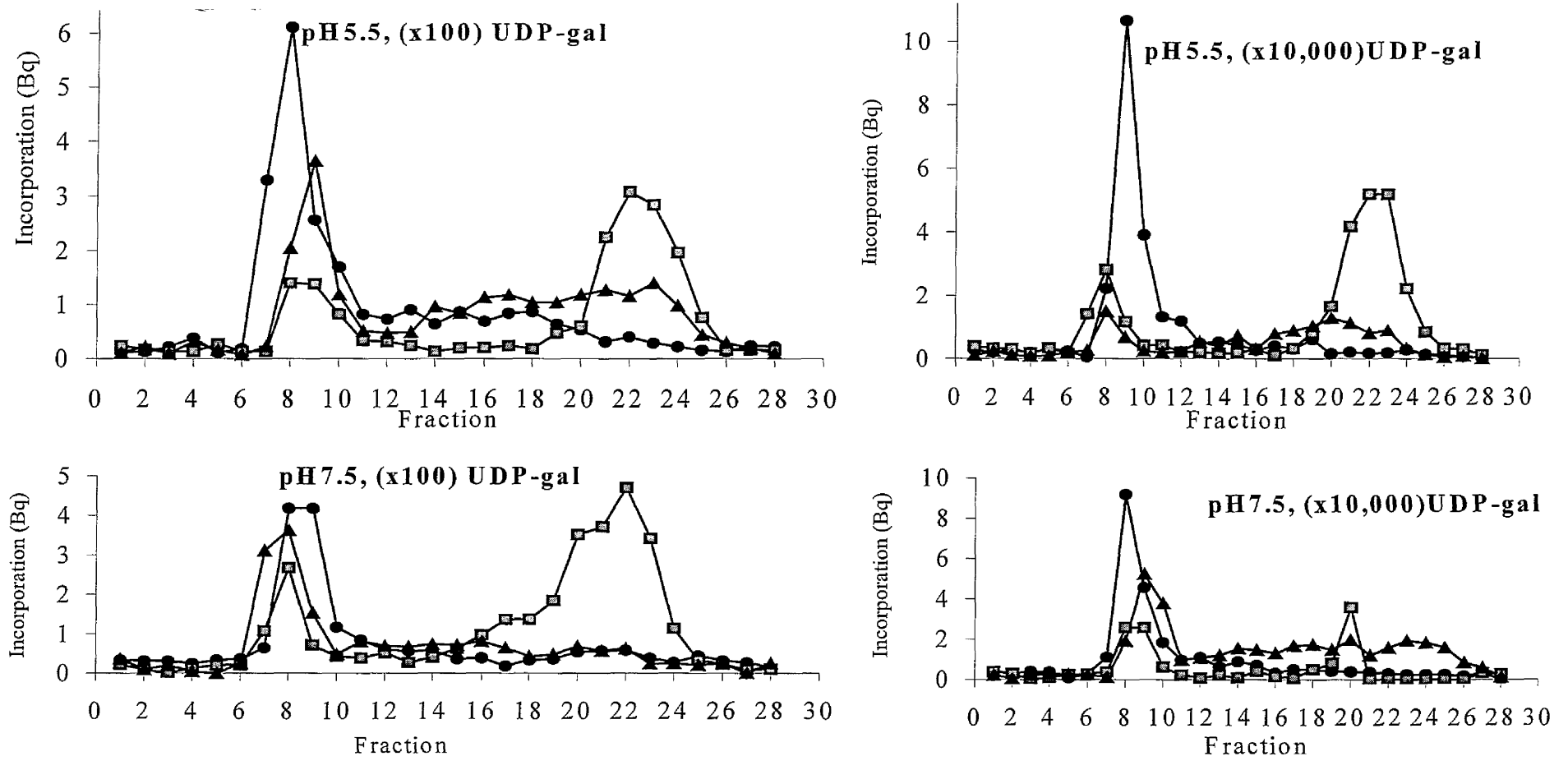

Fig 22. Gel filtration on Bio-Gel P10 column of the enzyme-treated high molecular weight EDTA extracts of different particulate enzyme preparations. Standard incubations were used, except that different concentrations of non-radioactive UDP-galactose and resuspension buffers at the specified pH(s) were used. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 20-26. Treated extracts are represented by circles: control (no enzyme); squares: galactanase-treated; and triangles polygalacturonase-treated.

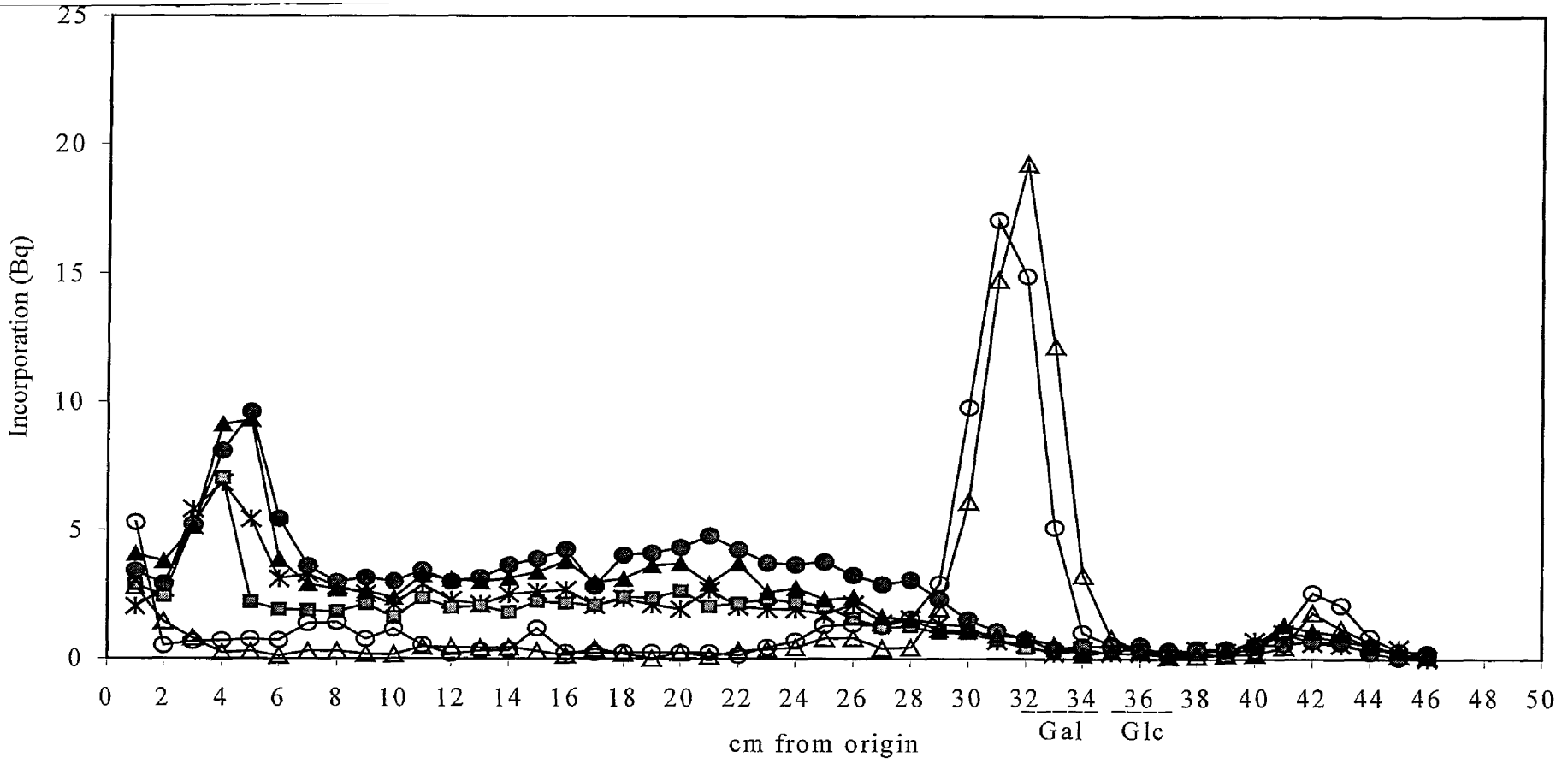

Fig 23. Paper chromatography of the solubilised material after different time intervals (0min, 30min, 60min, and 18hrs) of galactanase treatments of the particulate enzyme preparation. Controls (only buffer) were performed at 60min and 18hrs. The solubilised material was run for 18hrs on paper chromatography in a 10:4:3 EPW solvent system. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining. Crosses: galactanase treatment for 0min; squares: galactanase treatment for 30min; closed circles: galactanase treatment for 60min; closed triangles: galactanase treatment for 18hrs; open circles: control treatments for 60min; and open triangles: control treatments at 18hrs.

Table 13. Galactanase time course of particulate enzyme preparation. % Breakdown by endo-1,4-β-galactanase enzyme is calculated with respect to the preparation with zero minutes galactanase treatment. Control treatments contained sodium acetate buffer (pH4.5) at 40°C with no enzyme.

Time of treatment with endo-1,4-β-galactanase	Radioactivity in pellet after treatment (Bq)	%Breakdown
0 min	87.31 ± 2.20	----
15 min	66.29 ± 4.58	24%
30 min	64.13 ± 7.24	26%
60 min	58.00 ± 1.11	34%
18 hr	34.98 ± 7.00	60%
Control (buffer), 18 hr	71.82 ± 1.98	16%

the presence of a dimer and a trimer respectively. The peaks in fractions 4-6 might represent a tetramer. To try to avoid smearing, the galactanase buffer was desalted by running on Bio-Gel P4 column and collecting only the high molecular weight material. The concentrated, desalted enzyme was used to repeat the same experiment and two methods were applied to terminate the galactanase treatment either by boiling or by adding ethanol to a final concentration of 70%. As it is evident in Fig 24, desalting the enzyme did not result in the disappearance of smearing. This suggests that the enzyme itself was interfering with the chromatographic system.

7-Extracting with hot water

From galactanase time-course experiments, it was suspected that a low molecular weight contaminant existed that was most probably galactose. To test this further, washed particulate product (washed with 70% ethanol and with cold water) was extracted with water at 100°C for 10 minutes. The extract was then run on paper chromatogram in two systems, 8:2:1 and 10:4:3 (v/v) EPW, for 18 hours. In the former system [Fig 25] the galactose marker ran in fractions 11-13 and the glucose marker in fractions 13-15, while in the latter system [Fig 26] galactose ran in fractions 37-39 and glucose in fractions 39-41. These figures revealed that the main low molecular weight material was clearly galactose. There was an unidentified product that ran faster than galactose in both systems. Some of the material at the origin of the 8:2:1 (v/v) EPW system may have been UDP-galactose. Peaks also existed in the disaccharide region, suggesting the presence of galactobiose or other oligosaccharides. Hence such a wash could not be performed routinely, but it confirmed our suggestion that some galactose was present in the low molecular weight product.

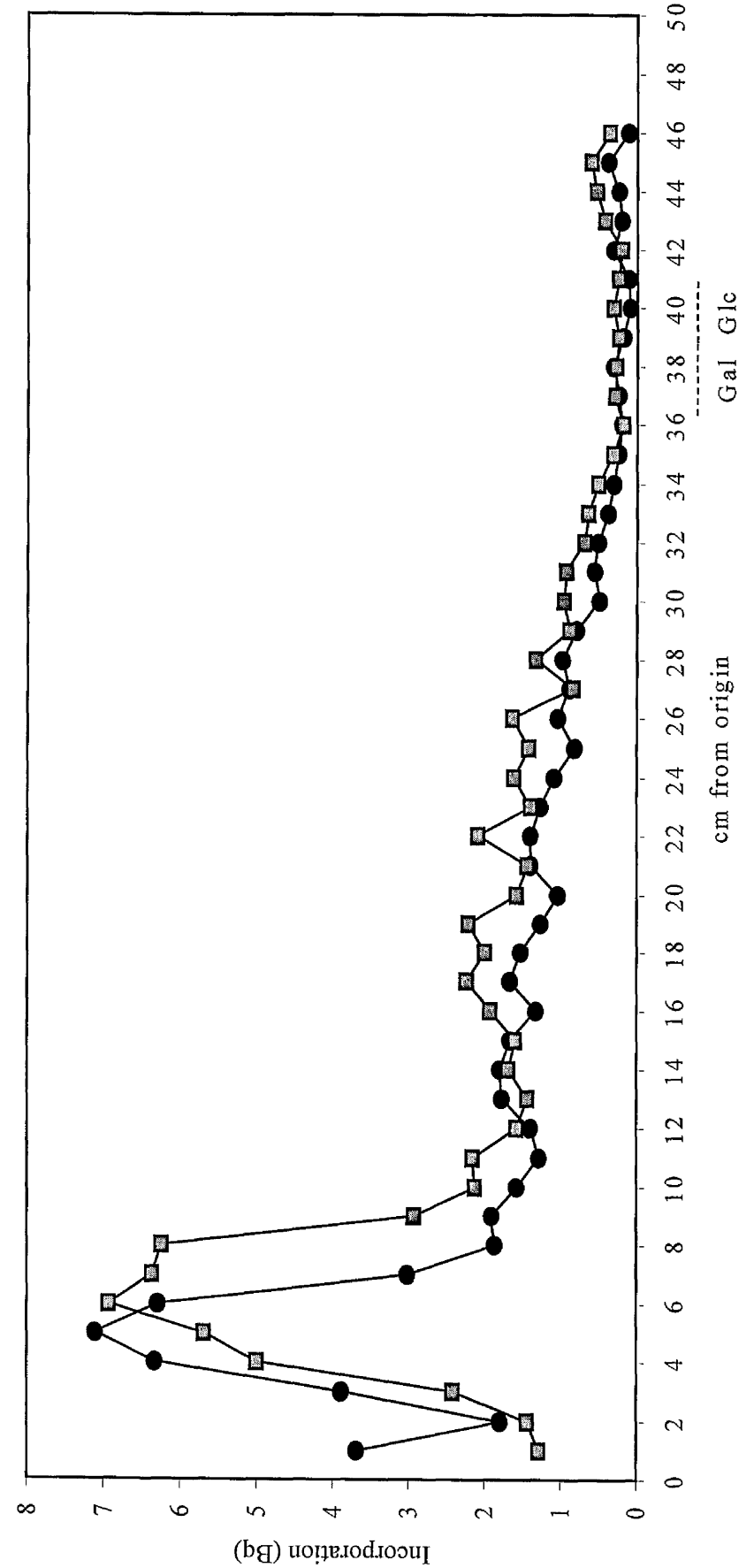

Fig 24. Paper chromatography of the solubilised material after galactanase treatment (18hr) of the particulate enzyme preparation with desalted enzyme. The 18hr treatment of Fig 23 was repeated after desalting and concentrating the galactanase enzyme on a Bio-Gel P 4 column to try to avoid smearing. The galactanase treatments were terminated either by boiling (represented by circles) or by adding ethanol to get a 70% final concentration (represented by squares). The solubilised material was run for 18hrs on paper chromatography in a 10:4:3 EPW solvent system. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining.

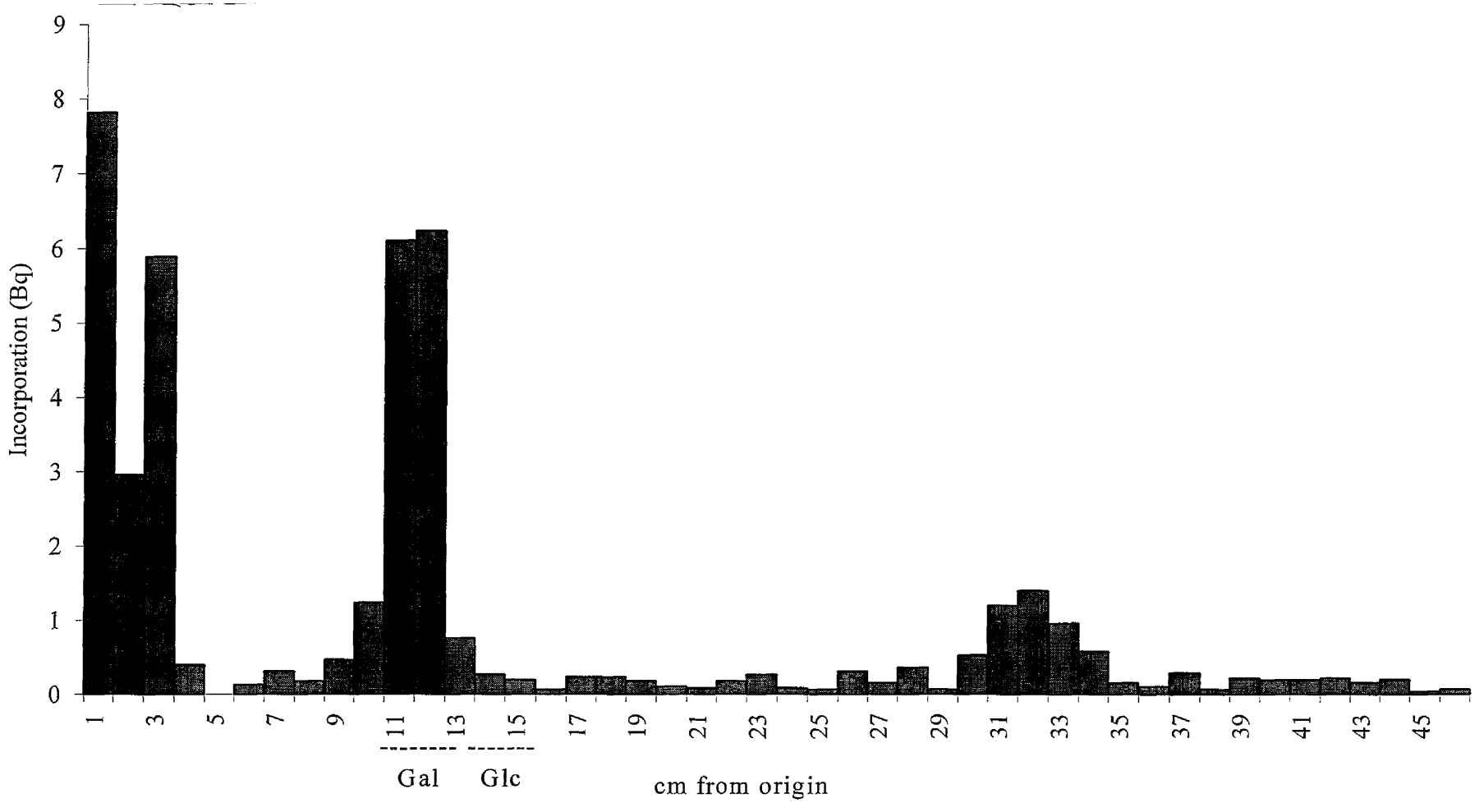

Fig 25. Descending paper chromatography, in 8:2:1 ethyl acetate: pyridine: water (v/v) system, of the extracts of the particulate enzyme preparations extracted with water at 100°C for 10min. The extracts were run on descending paper chromatography for 18 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining.

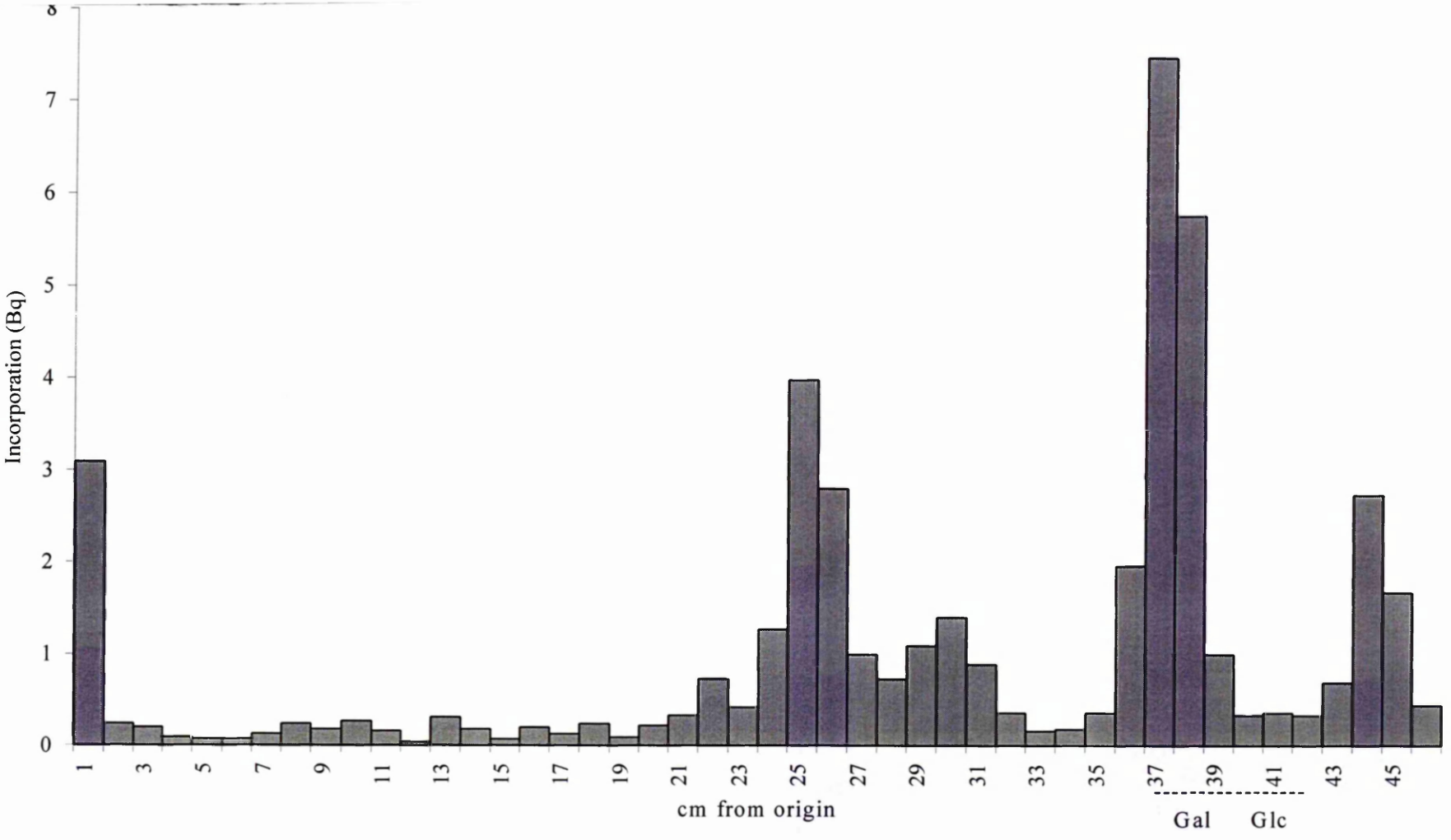

Fig 26. Descending paper chromatography, in 10:4:3 ethyl acetate: pyridine: water (v/v) system, of the extracts of the particulate enzyme preparations extracted with water at 100°C for 10min. The extracts were run on descending paper chromatography for 18 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining.

8-Effect of different additional washes

This study was performed in an attempt to find a combination of washes that decrease the low molecular weight contaminant from EDTA extract or galactanase solubilised material. It was already known that some of this low molecular weight contaminant was galactose. Thus after the standard ethanol and water washes, additional water washes were performed at 30°, 40°, or 50° C for 5 minutes or with 0.8 $mol.l^{-1}$ ammonium sulphate at 40°C (similar to that present in galactanase enzyme buffer) for 5 minutes. These washes were run on Bio-Gel P10 column [Fig 27]. Later the pellets washed with these additional washes or pellets as control (with a water wash at room temperature or no extra wash at all) were extracted with hot EDTA-phosphate buffer ($50mmol.l^{-1}$, pH6.8) and run on Bio-Gel P10 [Fig 28]. This investigation showed that all the additional washes seem to extract significant amounts of low molecular weight material, but ammonium sulphate extracted additional high molecular weight material whereas water at 40°C and 50°C extracted additional intermediate molecular weight material [Fig 27]. Thus it was better either to perform a water wash at room temperature or at temperatures not higher than 30°C or to perform no additional wash. Since the best EDTA extracts were obtained from the pellets with only standard washes [Fig 28], the rest of the experiments were carried out with only standard washes.

9-Effect of external acceptors

The effect of adding external acceptors such as galactan or pectin at different concentrations was further investigated to investigate whether their stimulatory effect (Chapter III, Table 2) was on the high molecular weight EDTA extract. From the previous chapter, it was clear that galactan resulted in the most stimulation while pectin

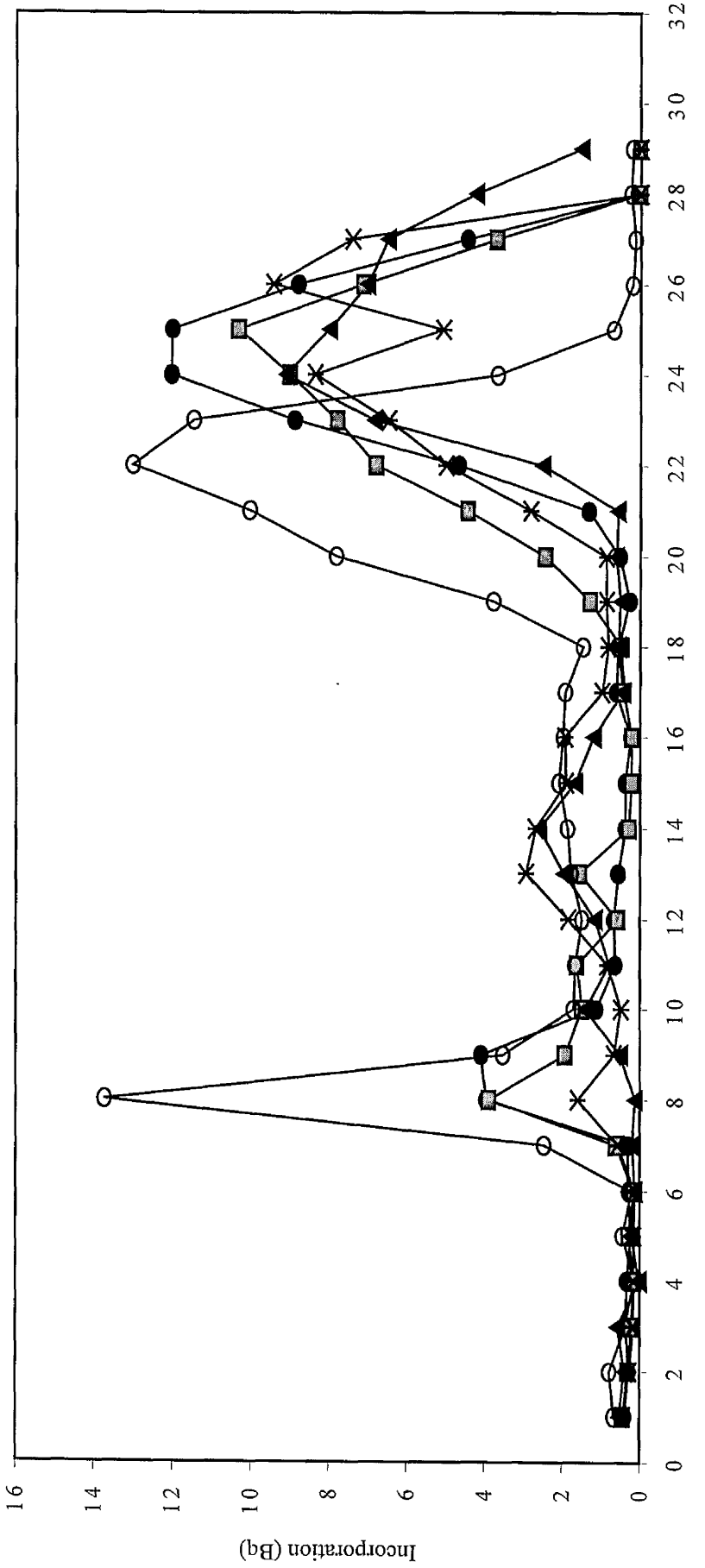

Fig 27. Gel filtration on Bio-Gel P10 column of additional washes of the particulate enzyme preparation. Additional water washes (at 30°, 40°, or 50°C) or washes with 8mol.l^{-1} ammonium sulphate were performed for 5 minutes to the particulate enzyme preparation after the standard washes. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 21-27. Closed circles: control (water wash at room temperature); squares: water wash at 30°C; triangles: water wash at 40°C; crosses: water wash at 50 °C; and open circles: ammonium sulphate washes.

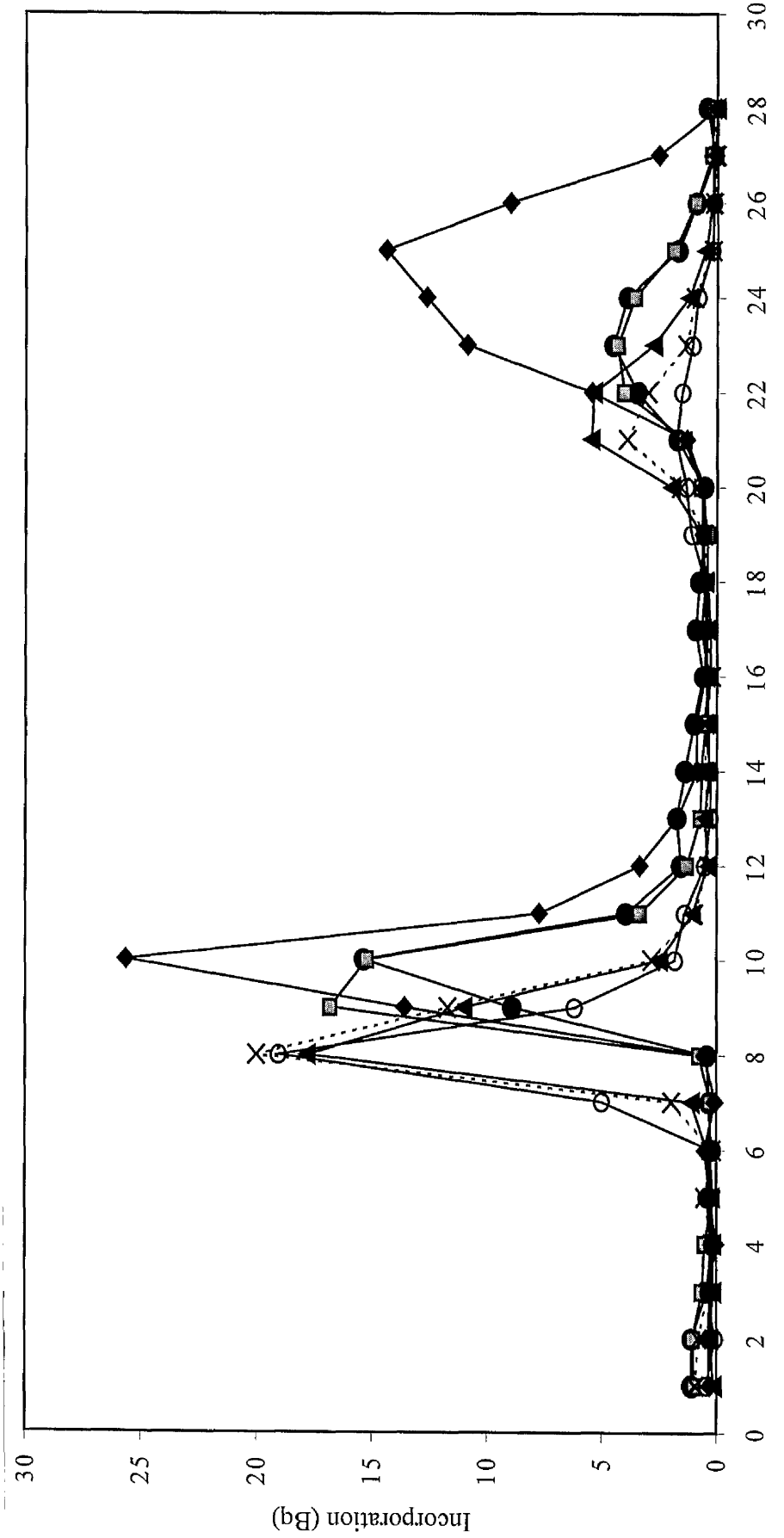

Fig 28. Gel filtration on Bio-Gel P10 column of EDTA extracts of the particulate enzyme preparation after the additional washes (stated in Fig 27). The washed particulate preparations were extracted with hot EDTA-phosphate at 100°C for 10 min. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 21-27. EDTA extracts were run from particulate product washed with water at room temperature (closed circles), water at 30°C (squares), water at 40°C (triangles), water at 50°C (crosses), ammonium sulphate washes (open circles), and extract from a preparation with only standard washes (diamonds).

had less effect. Different concentrations of galactan (0, 0.5, and 1% galactan) or pectin were added to the standard incubation. The incubations were terminated as usual with 90% ethanol and then washed (3x) with 70% ethanol only, then the EDTA extracts were run on Bio-Gel P10. No water wash was performed in order to avoid possible solubilisation of galactan in that wash. In such experiments there was no need to worry about the presence of low molecular weight material, as the main interest was in the high molecular weight product. Fig 29, shows that galactan did not cause much stimulation of the high molecular weight EDTA extract. On the contrary, high concentrations of galactan (1%) resulted in some inhibition (at least with the new particulate enzyme preparation conditions). Similarly, with exogenous pea pectin [Fig 30] or commercial pectin no real stimulation was observed. The pea pectin was prepared from EDTA extractions of cell wall preparations. Another trial was performed by preparing pea polysaccharide from the 4% KOH extract (after EDTA extractions) of cell wall preparations (see "Materials and Methods"). The effect of 1% (v/v) Triton X-100 was also tested in the presence or absence of the pea pectin or pea polysaccharide prepared by EDTA or KOH extraction of the cell wall respectively [Fig 31]. Triton X-100 is expected to allow the enzymes to be more accessible to acceptors. The results showed that the solubilisation of high molecular weight material was most with EDTA-pea pectin in the presence of Triton X-100, followed by KOH polysaccharide-pectin in the presence of Triton X-100, and then with Triton X-100 on its own. The rest of the acceptors behaved as the control (no external acceptor) or decreased solubilisation such as KOH-polysaccharide pectin. Thus it was found that most of the stimulation needed Triton X-100. To test the possibility if hydrolysed parts of galactan or pectin could act as acceptors, rather than the whole molecules, 1% galactan or 1% commercial pectin

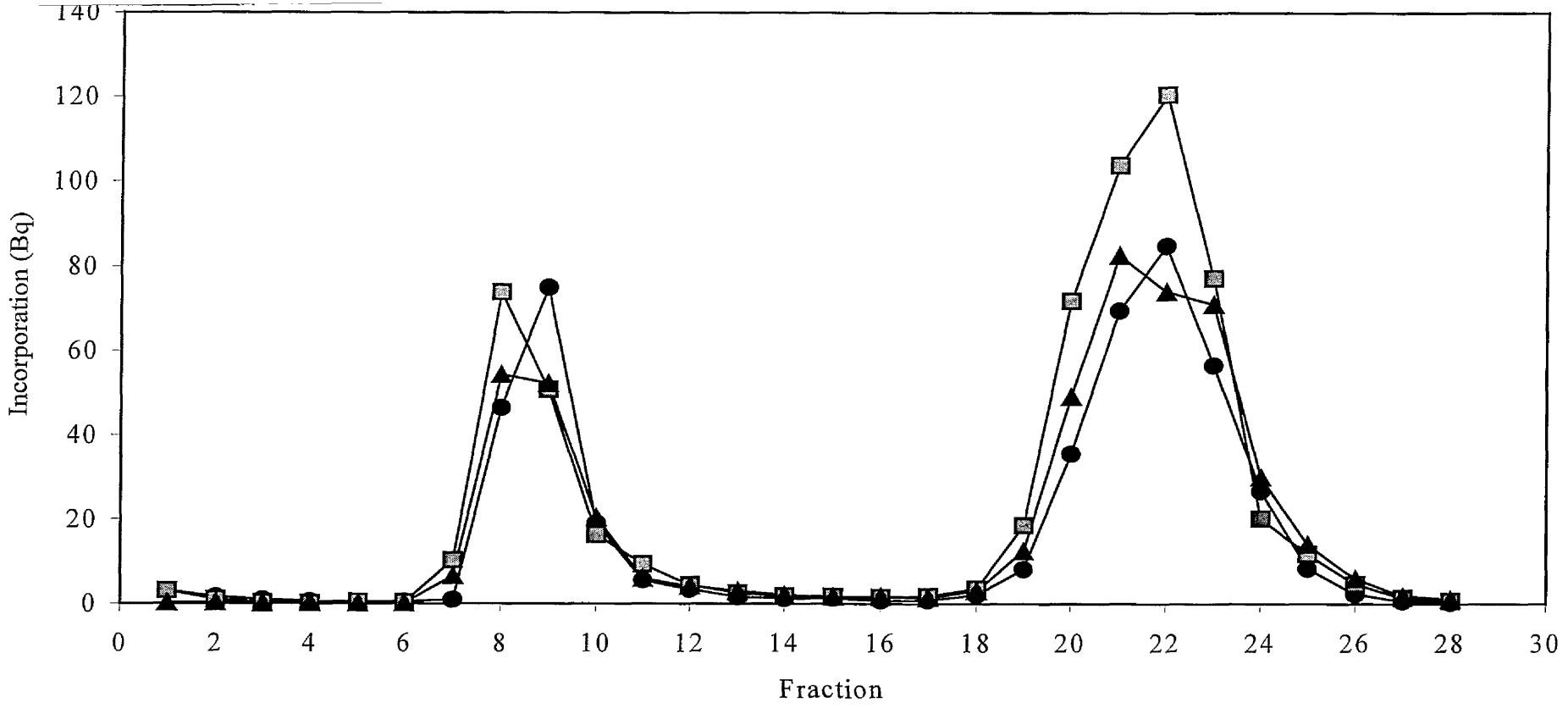

Fig 29. Gel filtration on Bio-Gel P10 column of the EDTA extracts of the particulate enzyme preparation after adding different concentrations of galactan. Standard incubations were carried out, except the additions of 0% (denoted by circles), 0.5% (denoted by squares), and 1% galactan (denoted by triangles). The incubations were terminated and washed with ethanol as the standard method except that no water wash was performed. They were next extracted with hot EDTA-phosphate and the extracts were run on Bio-Gel P10 column. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 21-26.

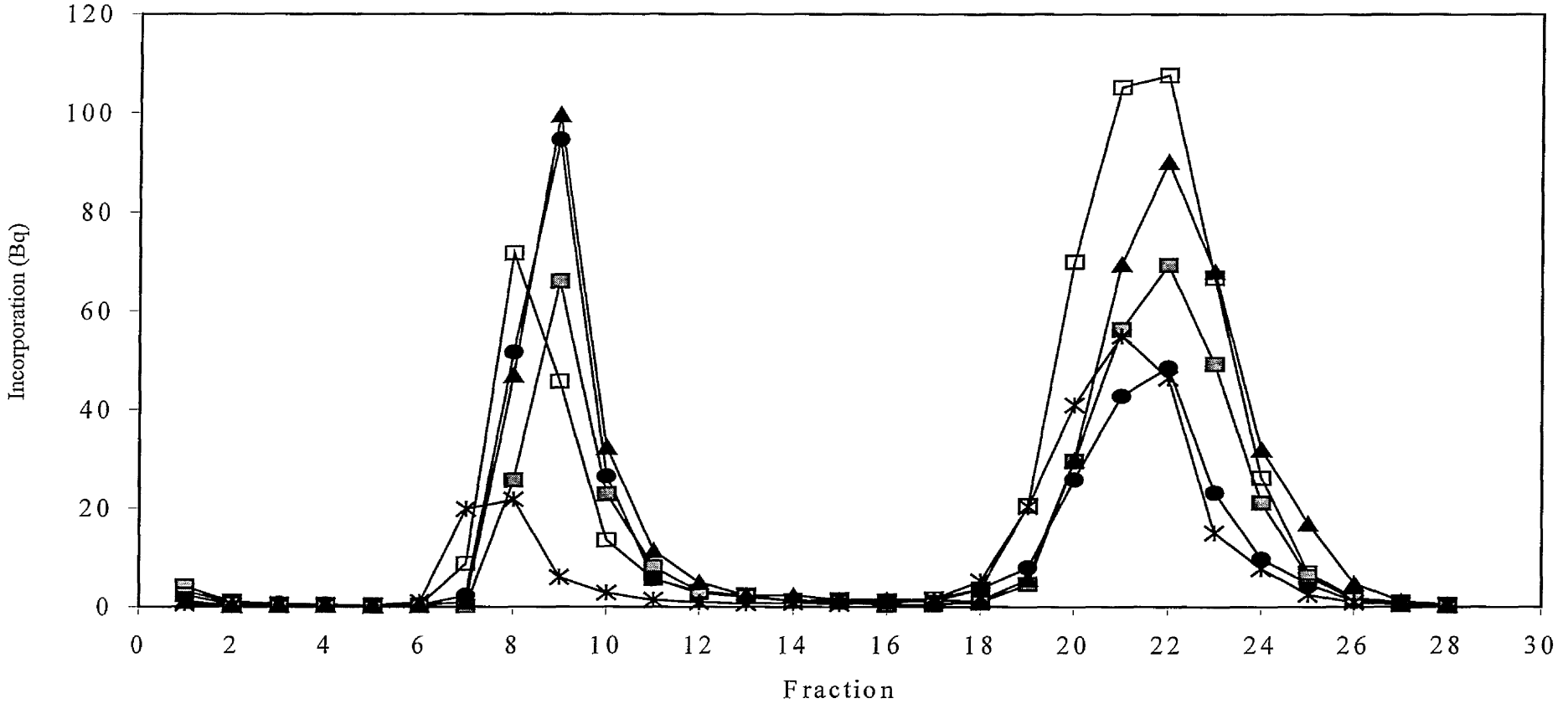

Fig 30. Gel filtration on Bio-Gel P10 column of the EDTA extracts of the particulate enzyme preparation after adding different concentrations of exogenous pea pectin and commercial pectin. Standard incubations were carried out, except the additions of 0µl (circles), 1µl (closed squares), 5µl (triangles), and 10µl (open squares) pea pectin to the particulate enzyme preparation. Commercial pectin, 10µl (crosses), was also added. Pea pectin was prepared from EDTA extractions of the cell wall. The incubations were terminated and washed with ethanol as the standard method except that no water wash was performed. They were next extracted with hot EDTA-phosphate. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 21-26.

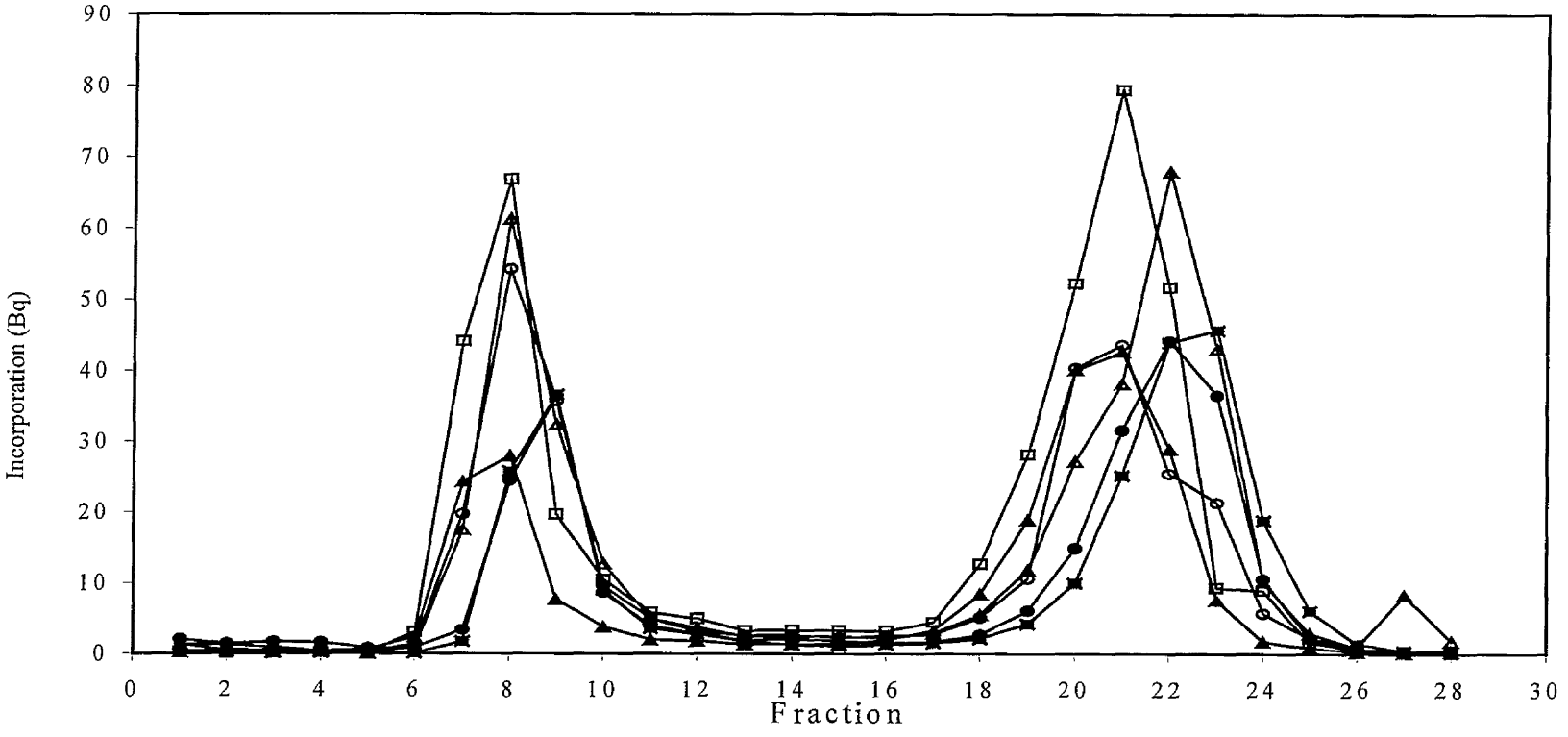

Fig 31. Gel filtration on Bio-Gel P10 column of the EDTA extracts of the particulate enzyme preparation after adding exogenous pea polysaccharide with or without Triton X- 100. Pea polysaccharides were prepared from EDTA or 4% KOH extractions of the cell wall. Standard incubations were carried out, except the additions of 10ul water (ctrl, denoted by closed circle), 1% Triton (denoted by open circles), EDTA-pea-pectin (denoted by closed squares), KOH-pea-polysaccharide (denoted by closed triangles), EDTA pea pectin in the presence of Triton X-100 (denoted by open squares), and KOH pea polysaccharide in the presence of Triton X-100 (denoted by open triangles). The incubations were terminated and washed with ethanol as the standard method except that no water wash was performed. They were next extracted with hot EDTA-phosphate and the extracts were run on Bio-Gel P10 column. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 20-26.

were treated with galactanase, polygalacturonase, or pectin lyase enzyme then added as acceptors [Fig 32, Fig 33]. Controls were formed by addition of the untreated substance or no external addition. There seemed to be no stimulation in the high molecular weight regions, though shifts are observed in the intermediate and low molecular weight regions.

Since the stimulation of galactan synthesis was not evident in the high molecular weight region, samples of galactan were hydrolysed with 0.1mol.l^{-1} TFA for 5 hours, concentrated, and added as external acceptors to the standard incubation. The particulate enzyme preparation was terminated with 90% ethanol and then analysed by paper chromatography using a 10:4:3 (v/v) EPW solvent system [Fig 34]. Compared to the results of Panayotatos and Villemez (1973), it appears that the TFA hydrolysed galactan was causing synthesis of galactobiose (Rgal=0.6), in which case galactose would have to be acting as an acceptor. This would be rather unusual for a polysaccharide synthase, but not impossible.

Thus the next logical step was to add galactose of different concentrations to the standard incubation, terminate with 90% ethanol and run this on a paper chromatogram using a 10:4:3 (v/v) EPW system. There was no stimulation due to galactose as compared to control [Fig 35].

B. Analysing the product under the new preparation conditions.

1-Total acid hydrolysis

The particulate enzyme preparation prepared under conditions specified in this chapter was hydrolysed by TFA (2mol.l^{-1} TFA at 120°C for 1 hour) and analysed by

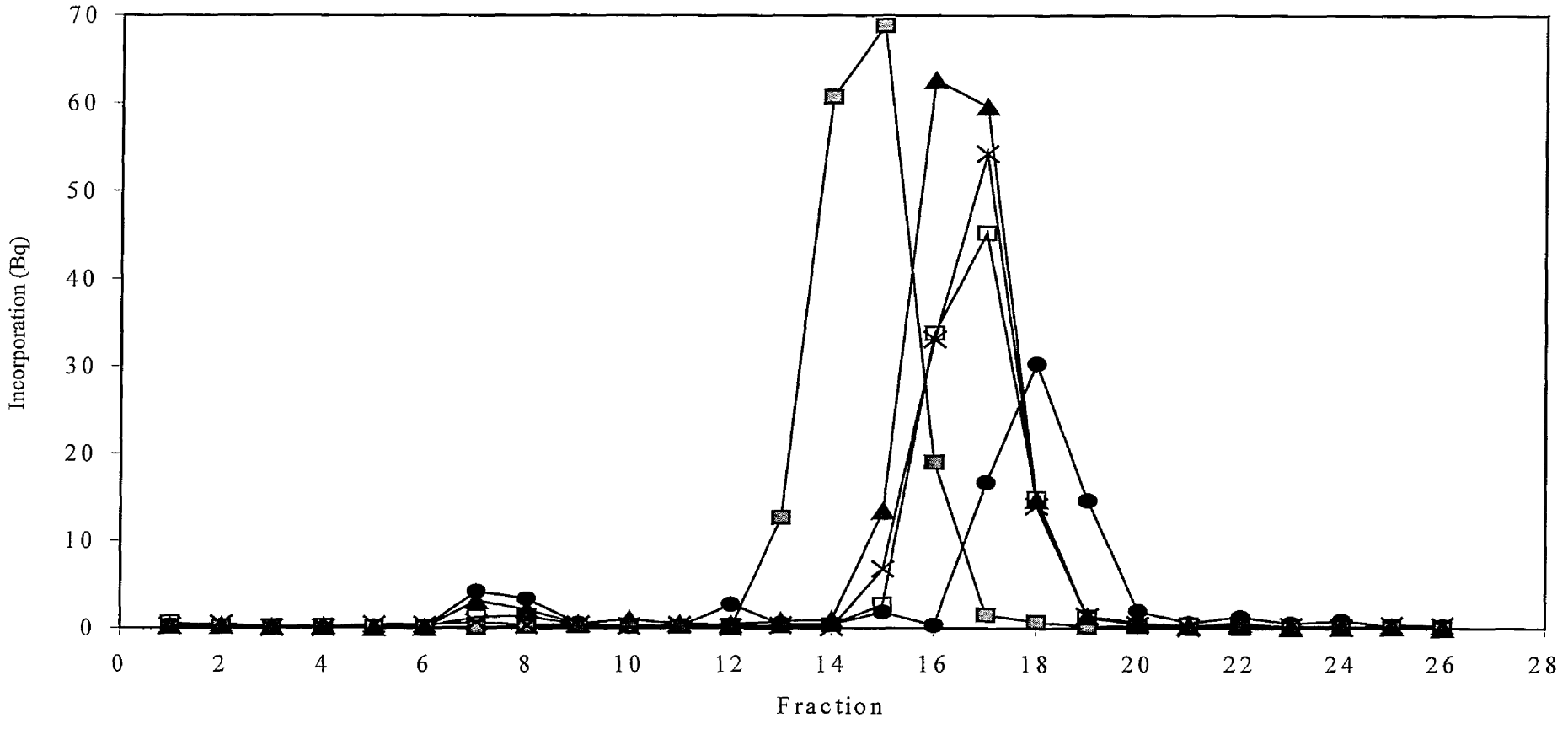

Fig 32. Gel filtration on Bio-Gel P10 column of the EDTA extracts of the particulate enzyme preparation after adding exogenous galactan hydrosylates. Commercial galactan (1%) was hydrolysed with galactanase, polygalacturonase, or pectin lyase before adding it to the standard incubation. Standard incubations were carried out, except the additions of 10ul water (ctrl, denoted by circles), 1% galactan (denoted by open squares), galactanase-treated galactan (denoted by closed squares), polygalacturonase-treated galactan (denoted by crosses), and pectin lyase-treated galactan (denoted by triangles). The incubations were terminated and washed with ethanol as the standard method except that no water wash was performed. They were next extracted with hot EDTA-phosphate and the extracts were run on Bio-Gel P10 column. The Blue Dextran marker ran between fractions 6-9, while the $CoCl_2$ marker ran between fractions 16-21.

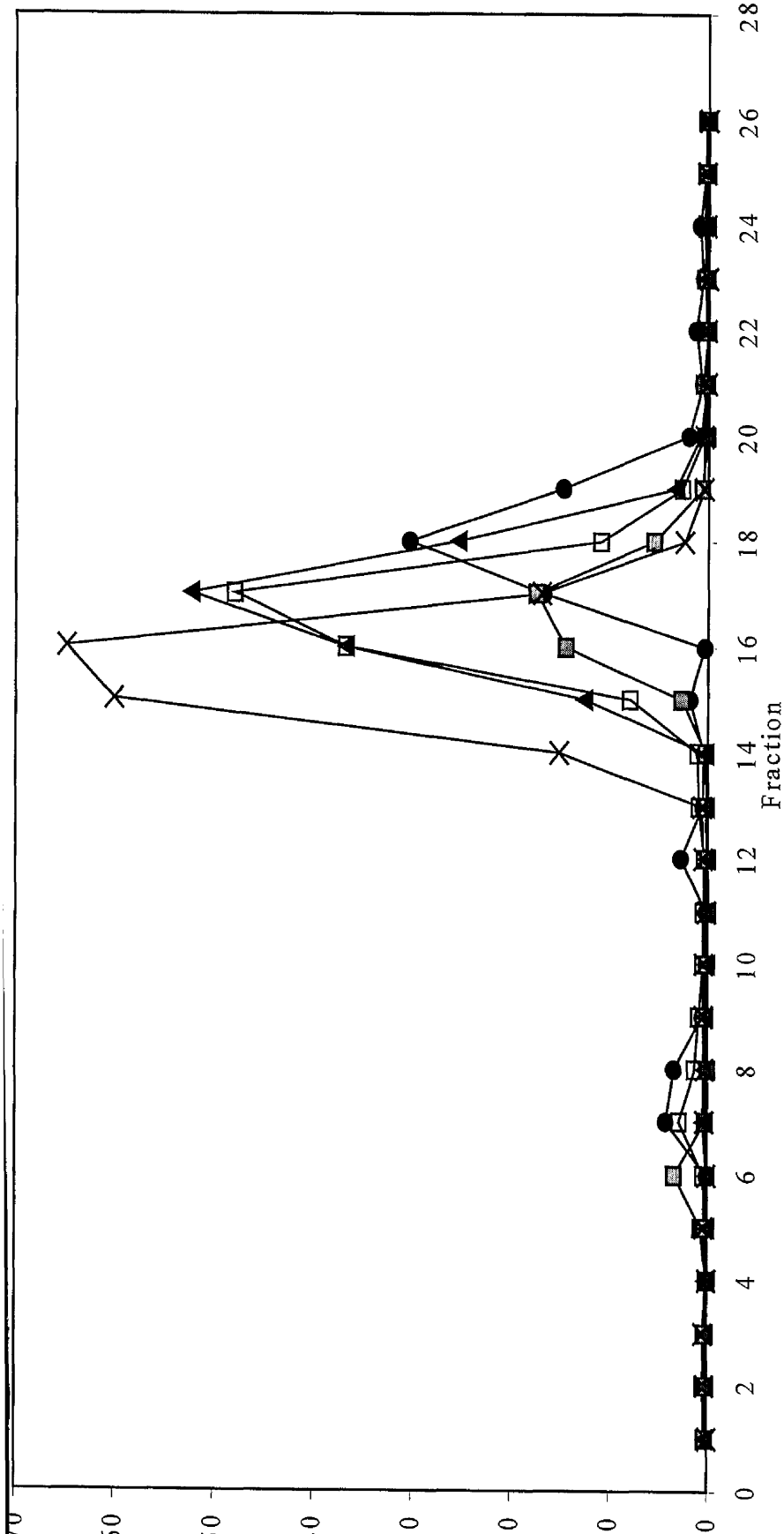

Fig 33. Gel filtration on Bio-Gel P10 column of the EDTA extracts of the particulate enzyme preparation after adding exogenous pectin hydrosylates. Commercial pectin (1%) was hydrolysed with galactanase, polygalacturonase, or pectin lyase before adding it to the standard incubation. Standard incubations were carried out, except the additions of 10ul water (ctrl, denoted by circles), 1% pectin (denoted by open squares), galactanase-treated pectin (denoted by closed squares), polygalacturonase-treated pectin (denoted by crosses), and pectin lyase-treated pectin (denoted by triangles). The incubations were terminated and washed with ethanol as the standard method except that no water wash was performed. They were next extracted with hot EDTA-phosphate and the extracts were run on Bio-Gel P10 column. The Blue Dextran marker ran between fractions 6-9, while the $CoCl_2$ marker ran between fractions 16-21.

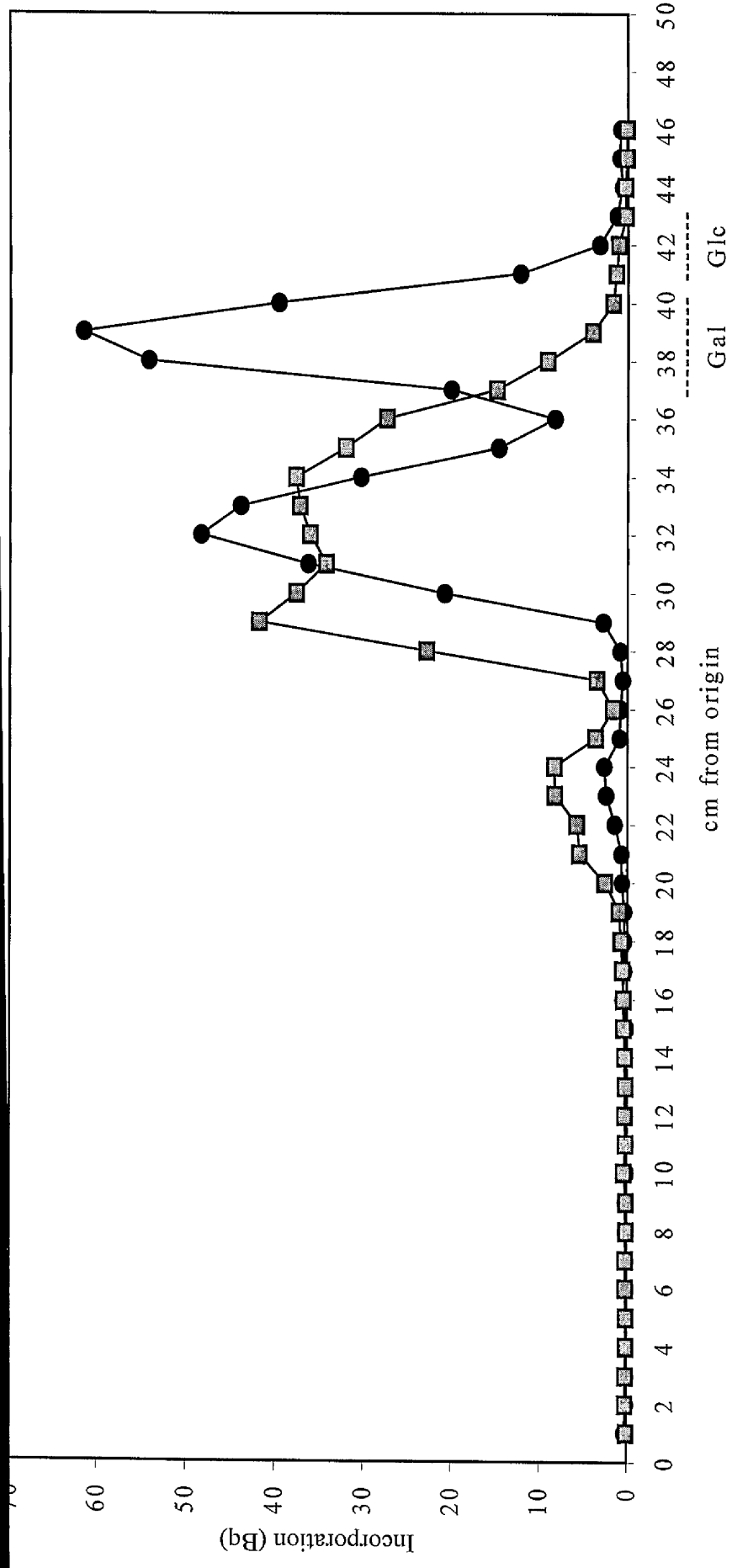

Fig 34. Descending paper chromatography of the 90% ethanol used in terminating the incubations of the particulate enzyme preparation where hydrolysed galactan was added. Standard incubations were carried out, except the addition of hydrolysed galactan (hydrolysed by 0.1mol.l^{-1} TFA for 5 hrs). The particulate enzyme preparation was terminated with 90% ethanol, which was run on paper chromatography in 10:4:3 ethyl acetate: pyridine: water (v/v) system for 18 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining. The 90% ethanol from the incubation with hydrolysed galactan is represented by squares, whereas the control (no additions) is represented by circles. Radioactive UDP-galactose ran in fractions [30-34].

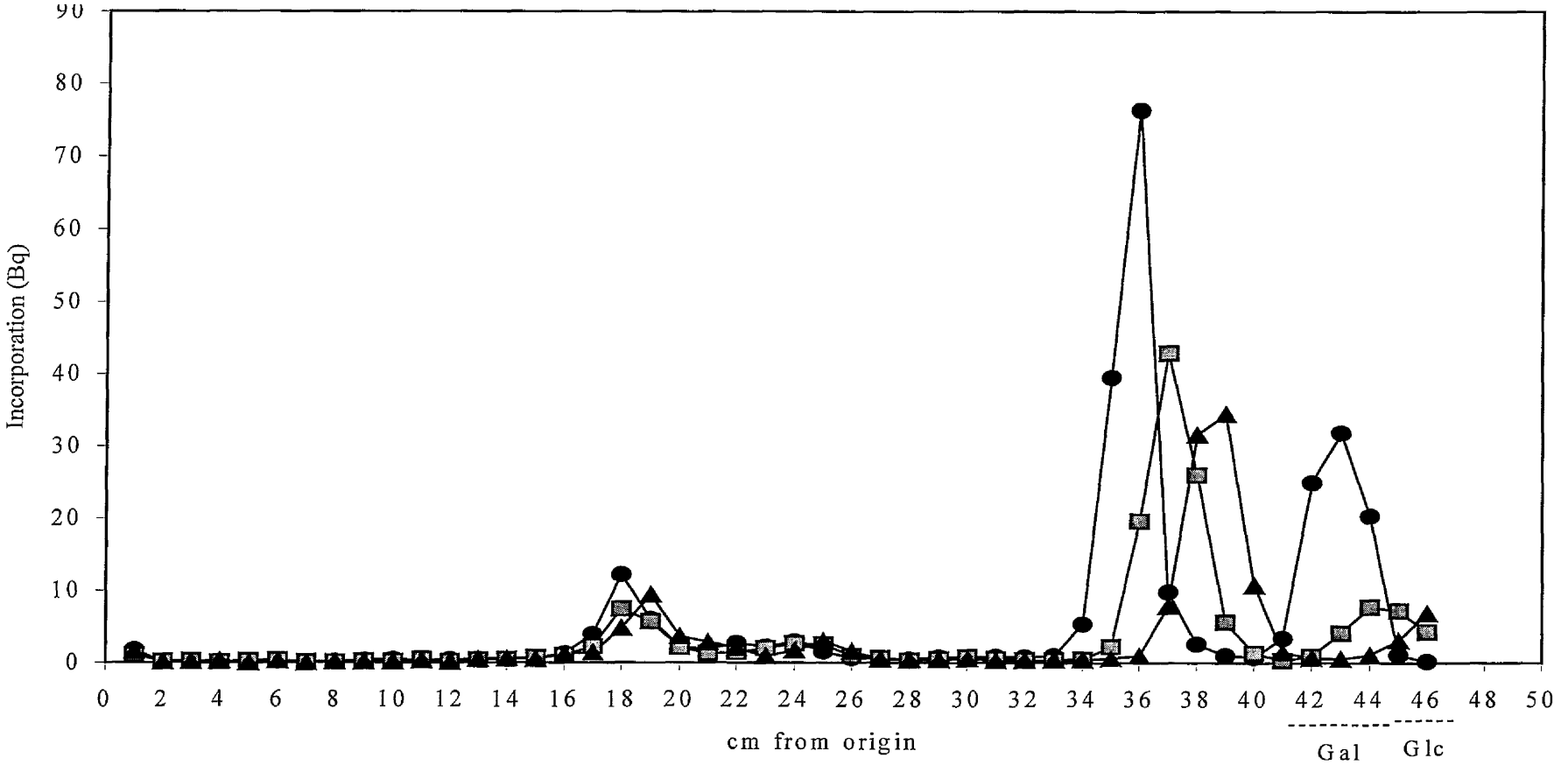

Fig 35. Descending paper chromatography of the 90% ethanol used in terminating the incubations of the particulate enzyme preparation where galactose of different concentrations was added. Standard incubations were carried out, except the addition of galactose of different concentrations. The particulate enzyme preparation was terminated with 90% ethanol, which was run on paper chromatography in 10:4:3 ethyl acetate: pyridine: water (v/v) system for 18 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining. The 90% ethanol from the incubations with 0mmol.l^{-1} (control: circles), 10mmol.l^{-1} (squares) and 100mmol.l^{-1} galactose (triangles) are shown. Radioactive UDP-galactose ran in fractions [37-40].

paper chromatography in 8:2:1 (v/v) EPW. This revealed a major peak that ran parallel to the marker sugar galactose confirming that the radioactive label was in galactose [Fig 36]. A second peak also existed that corresponded to arabinose, which ran twice as fast as the galactose marker.

2-Partial acid hydrolysis

The particulate enzyme material prepared under conditions specified in this chapter was subjected to partial acid hydrolysis by TFA (0.1mol.l^{-1} TFA at 100°C for 3 hours) and analysed by paper chromatography in 10:4:3 (v/v) EPW [Fig 37] for 18 hours. Galactose and glucose markers ran in fractions 29-32 and 32-35 respectively as shown by silver nitrate staining. The Rgal values obtained [Fig 37] revealed peaks that corresponded to the products obtained by Panayotatos and Villemez (1973) of hexamer, pentamer, trimer, dimer (galactobiose), galactose, and glucose [Table 14] confirming the presence of a β-1,4-galactan product.

3-Extractions and enzyme treatments

a-EDTA and KOH extractions:

The particulate enzyme preparation prepared under conditions specified in this chapter was extracted with hot EDTA-phosphate buffer (50mmol.l^{-1}, pH6.8) or with 4% KOH (in the presence of 0.1% $NaBH_4$) to verify that a high molecular weight product similar to that in the previous chapter was produced. The extractions were neutralised and concentrated by roto-evaporation then run on Bio-Gel P10 column [Fig 38]. This system solubilised more EDTA extract in the high molecular weight region than with

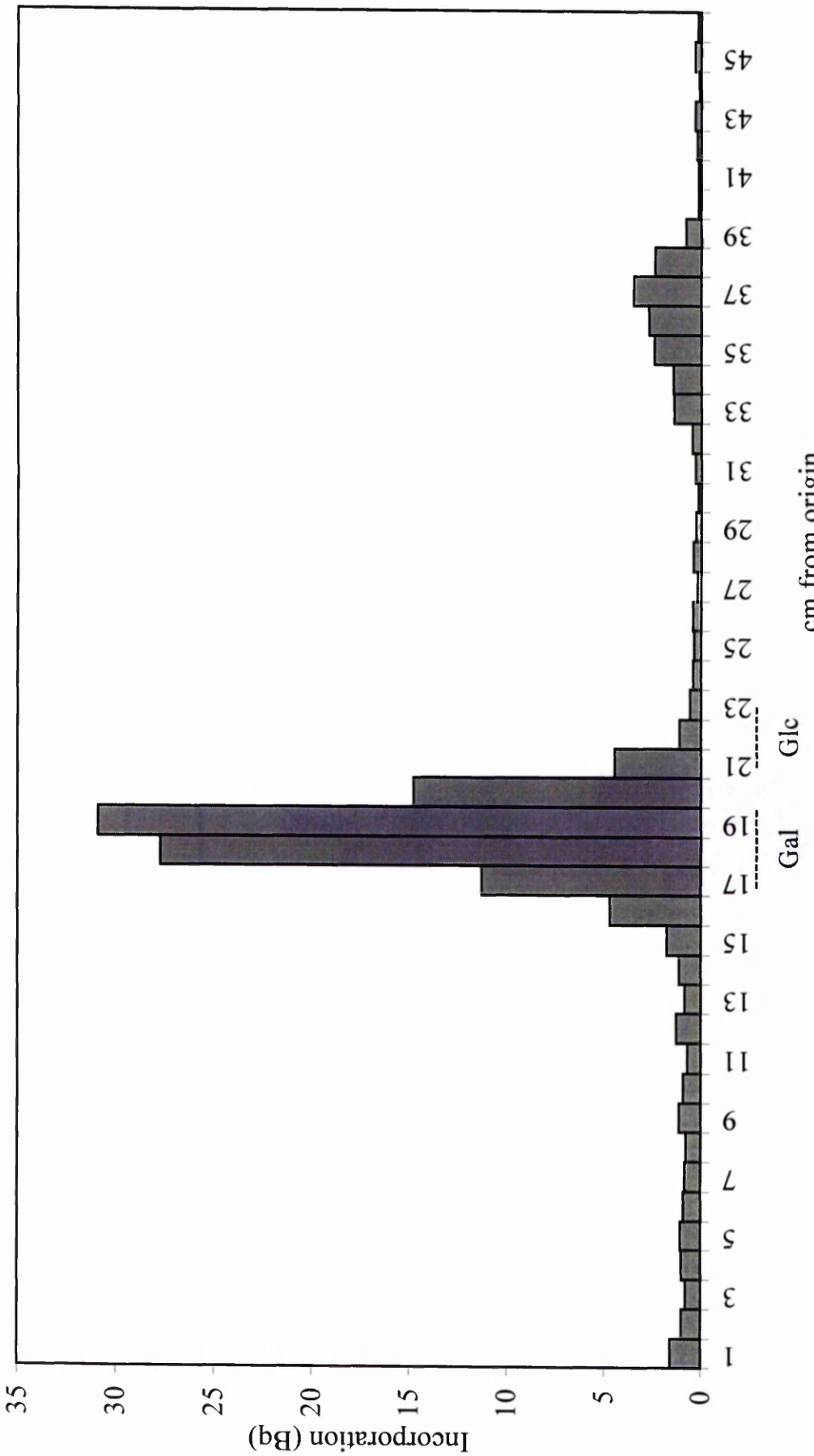

Fig 36. Descending paper chromatography of the complete TFA hydrolysate of the particulate enzyme product (prepared under new conditions) in 8:2:1 ethyl acetate: pyridine: water (v/v) system for 36 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining.

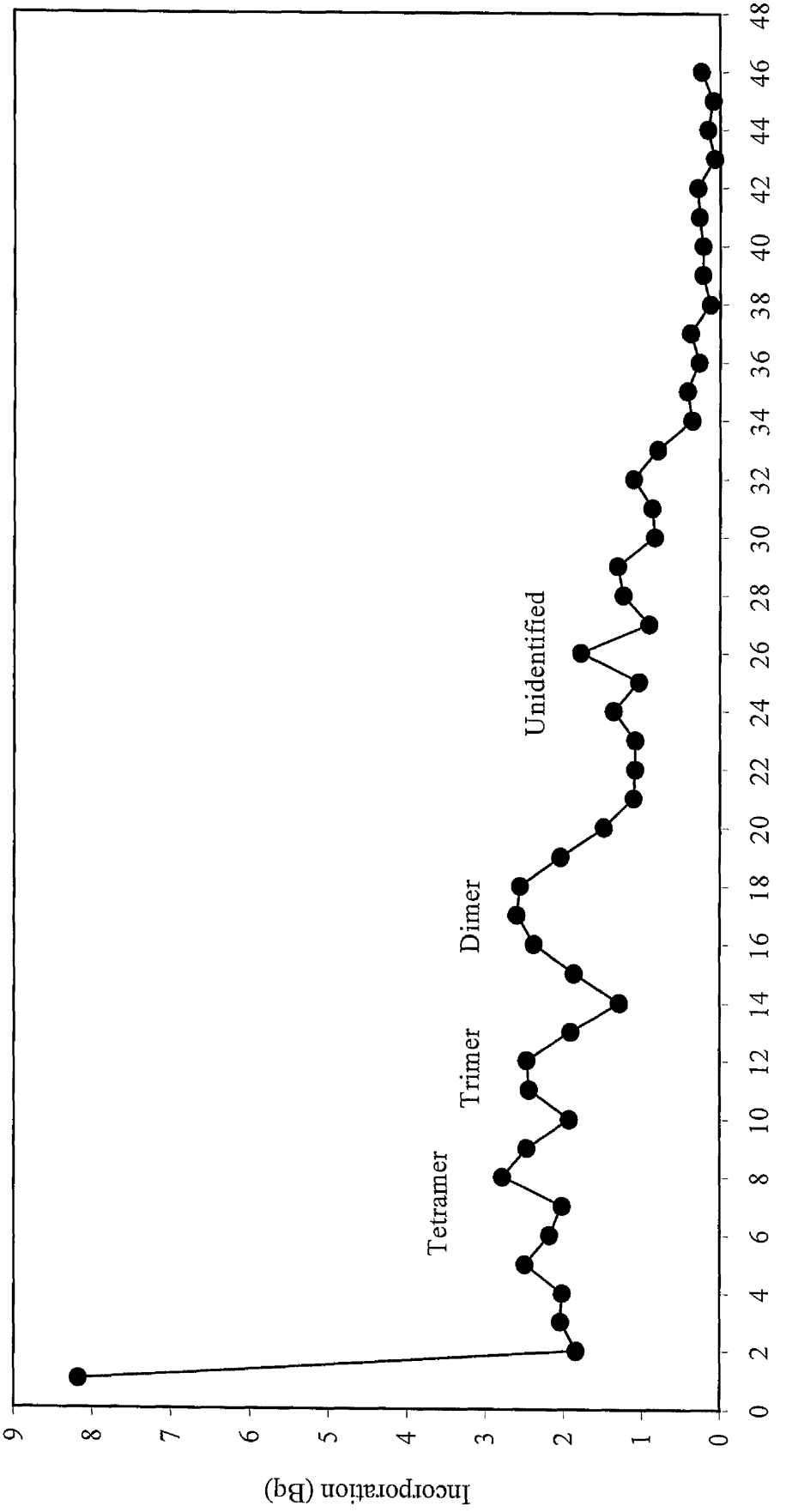

Fig 37. Descending paper chromatography of the partial TFA hydrolysate of the particulate enzyme product (prepared under new conditions) in 10:4:3 ethyl acetate: pyridine: water (v/v) system for 18 hrs. The particulate enzyme preparation was subjected to partial TFA hydrolysis for 3 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining. The peaks were identified in Table 8 as tetramers, trimers, and dimers of galactose.

Table 14. Comparison of Rgal values obtained by Panayotatos and Villemez (1973) in *Phaseolus aureus* hypocotyls and Rgal values obtained by partial acid hydrolysis of particulate enzyme material prepared from pea (*Pisum sativum*) epicotyls [Fig 37]. Rgal values are obtained by dividing distance travelled by solute (or peak) over distance travelled by galactose in our system. n indicates the degree of polymerisation of the oligosaccharide. Fraction corresponds to the position of the peak in Fig 37 whereas band corresponds to the band number observed in Panayotatos and Villemez (1973).

Peak	Panayotatos & Villemez (1973)		Rgal for partial TFA of product (3hrs) Fig 37		Product as compared to Panayotatos & Villemez (1973)
	Rgal	n	Rgal	n	
Fraction 1 or band 1	Rgal<0.2	n>4	0.03	n>4	Hexa- or penta- saccharide
Fraction 5 or band 2	Rgal<0.2	n>4	0.16	n>4	Hexa- or penta- saccharide
Fraction 8 or band 3	0.25	n=4	0.24	n=4	Tetrasaccharide
Fraction 12 or band 4	0.40	n=3	0.39	n=3	Trisaccharide, galactotriose
Fraction 18 or band 5	0.58	n=2	0.56	n=2	Disaccharide, galactobiose
Fraction 24, 26	-----		0.75, 0.81	unidentified	-----
Fraction 29 or band 6	0.90	unidentified	0.91	unidentified	-----
Fraction 32 or band 7	1.00	n=1	1.00	n=1	Galactose
Fraction 34 or band 8	1.20		-----		Glucose

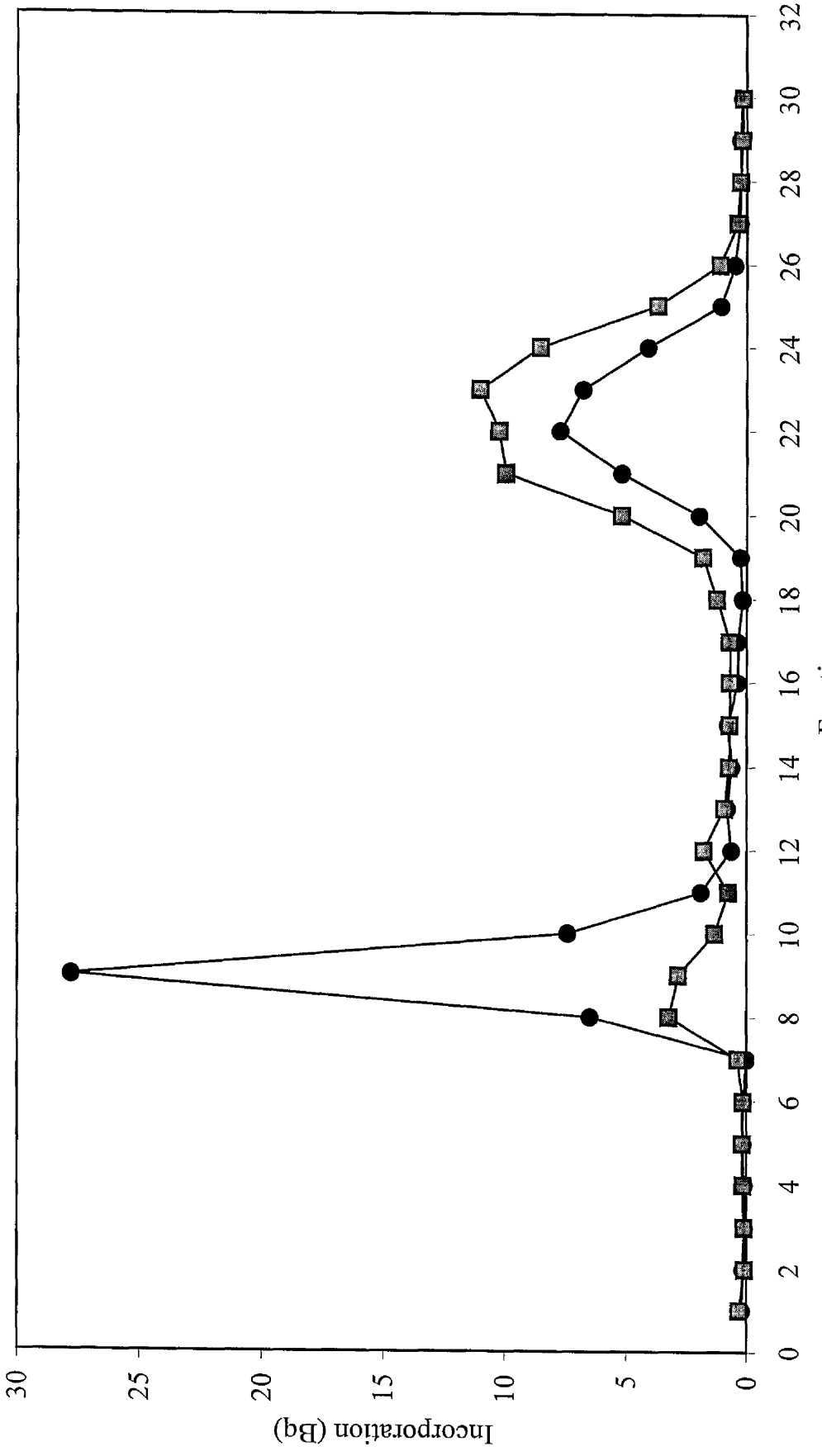

Fig 38. Gel filtration on Bio-Gel P10 column after extracting the particulate enzyme preparation (prepared under new conditions) consecutively with hot EDTA-phosphate followed by 4% KOH/0.1% NaBH4. Circles represent the EDTA-phosphate extract while squares represent the material extracted by 4%KOH. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 20-26.

the conditions used before and therefore was more appropriate for enzyme treatments and for studying the resulting shifts on Bio-Gel P10 columns.

b-Galactanase Treatment

Galactanase treatment of the high molecular weight EDTA extract [Fig 39] gave a large shift into a low molecular weight product. However, the control (which was incubated with sodium acetate buffer, pH 4.5, 40°C, 18 hours) gave a product of intermediate molecular weight that was not observed before. Hence an endogenous enzyme might have been breaking down our product in the 18 hours incubations, a possibility which was further investigated as will be shown later.

The high molecular weight EDTA extracts were treated with desalted galactanase enzyme or control (no enzyme added) and the low molecular weight product was collected, concentrated, and run on 10:4:3 (v/v) EPW system. As Fig 40 indicates, the low molecular weight galactanase-treated material had a large peak in the galactobiose region (fractions19-23) that was lacking in the control.

c-Enzyme Treatments

Total EDTA extracts were treated with galactanase or other enzymes, then run on a Bio-Gel P10 column [Fig 41] or on a Sepharose CL-6B column [Fig 42] to compare the changes in the molecular weight. The high molecular weight EDTA soluble material was excluded from Sepharose CL-6B. The EDTA extracts on Bio-Gel P10 show a large breakdown of our product by galactanase. Pectin lyase and endo-1,4-β-glucanase break the high molecular weight product into intermediate molecular weight on Bio-Gel P10. Thus the EDTA extract includes a galactan or a galactan

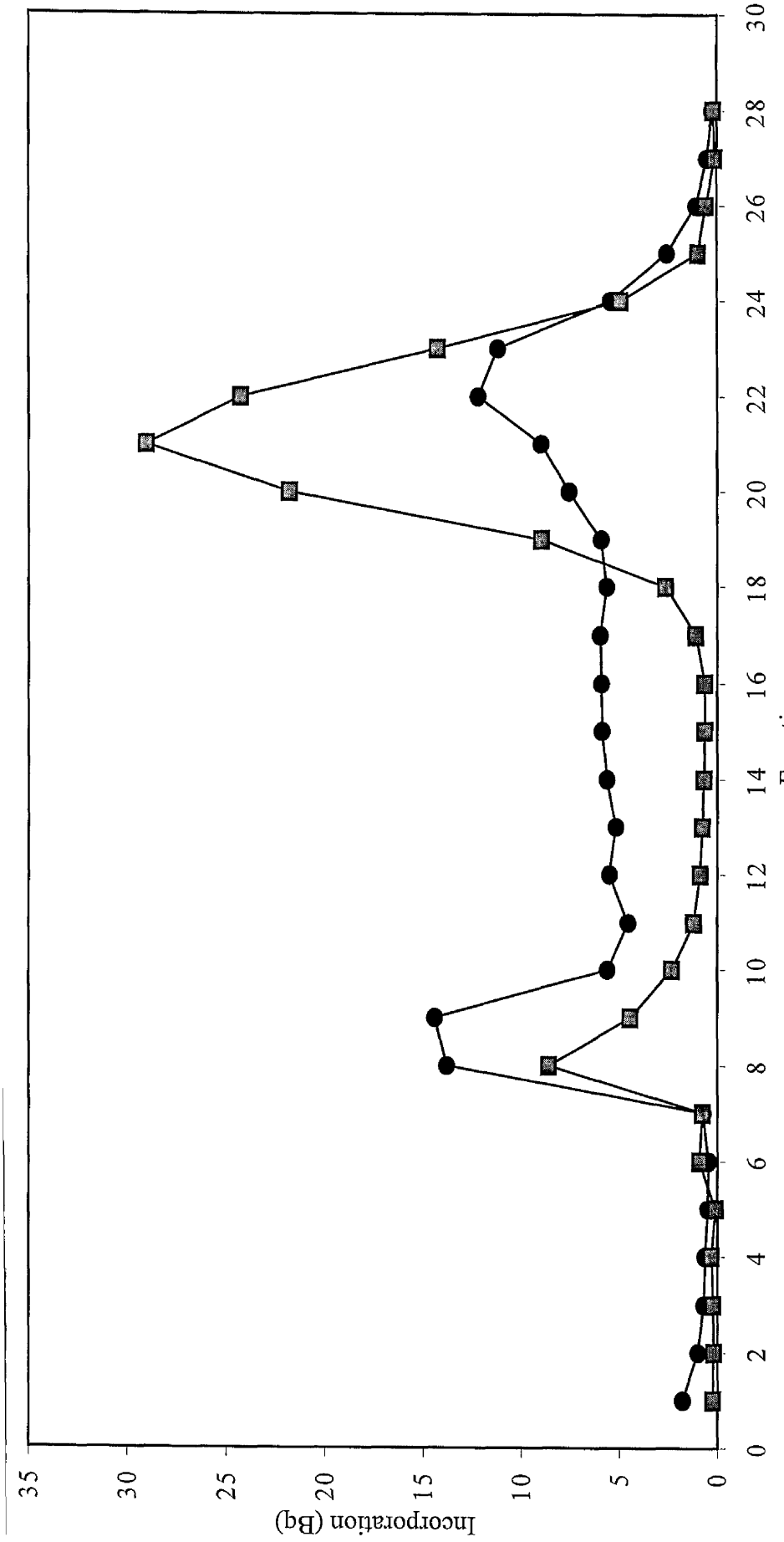

Fig 39. Gel filtration on Bio-Gel P10 column of the galactanase-treated (or control) high molecular weight EDTA extract. The high molecular weight EDTA extract was collected by running the EDTA extract from the particulate enzyme preparation (prepared under new conditions) on Bio-Gel P10 column. The high molecular weight material was next treated with galactanase enzyme or buffer for control (sodium acetate, pH 4.5) at 40°C for 18 hrs, then run on Bio-Gel P10. Circles represent the control while squares represent the galactanase-treated material. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 20-26.

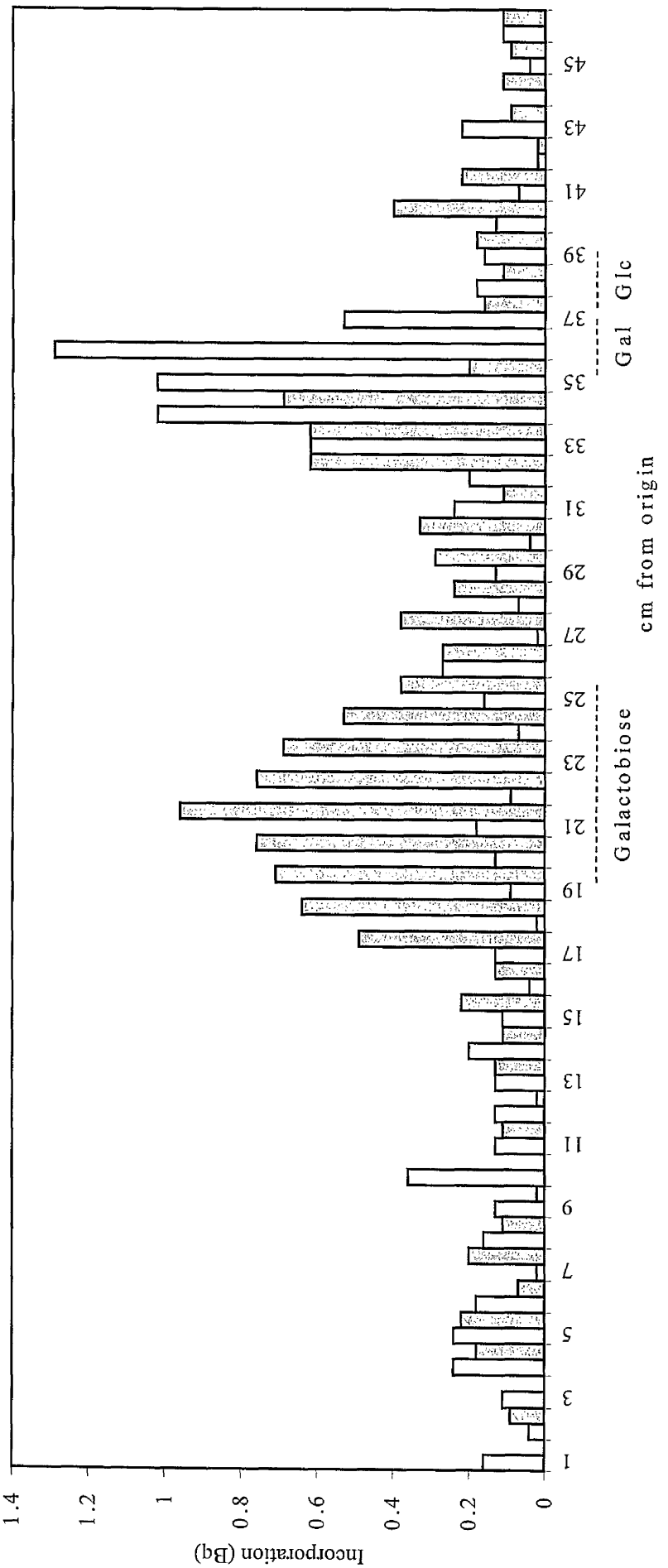

Fig 40. Descending paper chromatography of the low molecular weight fractions from the galactanase-treated (or ctrl) high molecular weight EDTA extract of the particulate enzyme material. The high molecular weight EDTA extract of the particulate enzyme preparation was treated with galactanase enzyme or control (buffer, no enzyme added) and run on Bio-Gel P10 column as in Fig 39. The low molecular weight material (in the $CoCl_2$ region) was collected and run on paper chromatography in 10:4:3 ethyl acetate: pyridine: water (v/v) system for 18 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining. Galactobiose region was determined by comparing Rgal values. UDP-galactose ran in fractions 32-34. The low molecular weight material from the galactanase-treated sample are denoted by bars with patterns, whereas the control is denoted by bars without patterns.

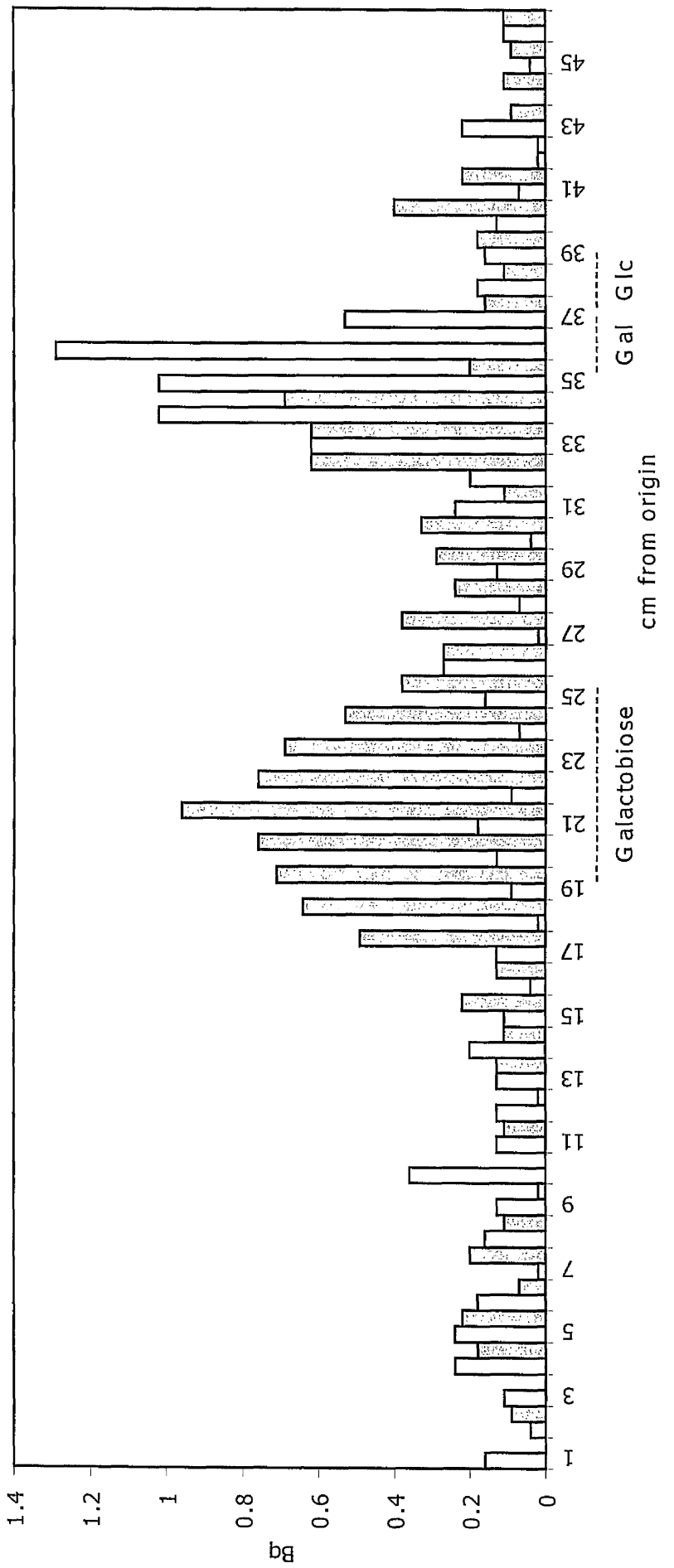

Fig 40. Descending paper chromatography of the low molecular weight fractions from the galactanase-treated (or ctrl) high molecular weight EDTA extract of the particulate enzyme material. The high molecular weight EDTA extract of the particulate enzyme preparation was treated with galactanase enzyme or control (buffer, no enzyme added) and run on Bio-Gel P10 column as in Fig 39. The low molecular weight material (in the $CoCl_2$ region) was collected and run on paper chromatography in 10:4:3 ethyl acetate: pyridine: water (v/v) system for 18 hrs. The positions for marker sugars galactose (Gal) and glucose (Glc) were determined by silver staining. Galactobiose region was determined by comparing Rgal values. UDP-galactose ran in fractions 32-34. The low molecular weight material from the galactanase-treated sample are denoted by bars with patterns, whereas the control is denoted by bars without patterns.

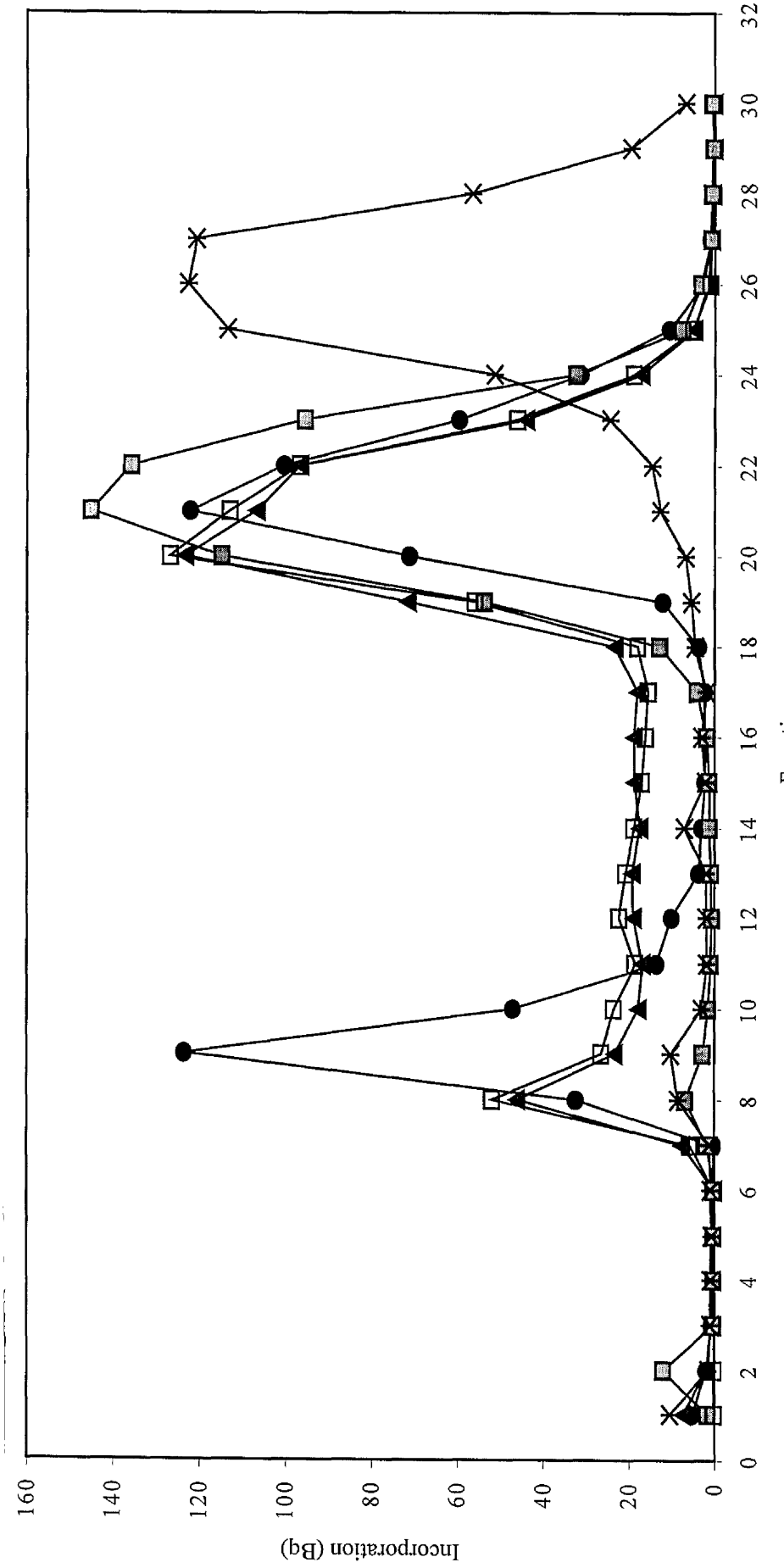

Fig 41. Gel filtration on Bio-Gel P10 column after treating the EDTA extracts of the particulate enzyme material (prepared under the new conditions) with different enzymes. The control consists of untreated extract only. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 20-26. [Closed squares: endo-1,4-β-galactanase treated extracts; open squares: pectin lyase treated; triangles: endo-1,4-β-glucanase treated; crosses: proteinase K treated; and circles: control treated extracts.]

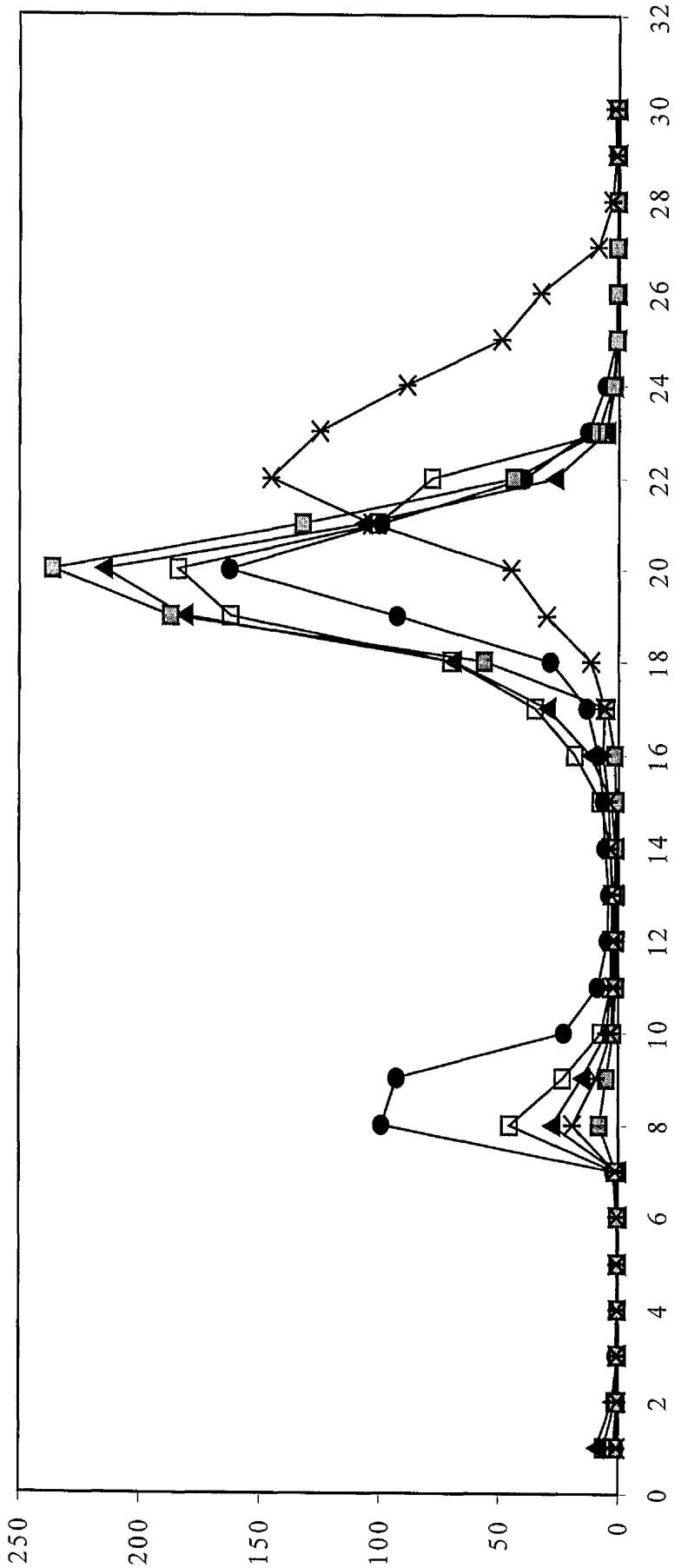

Fig 42. Gel filtration on Sepharose CL-6B column after treating the EDTA extracts of the particulate enzyme material (prepared under the new conditions) with different enzymes. The control consists of untreated extract only. The Blue Dextran marker ran between fractions 7-11, while the $CoCl_2$ marker ran between fractions 19-22. [Closed squares: endo-1,4-β-galactanase treated extracts; open squares: pectin lyase treated; triangles: endo-1,4-β-glucanase treated; crosses: proteinase K treated, and circles: control treated extracts.]

attached to a pectin backbone. Degradation by endo-1,4-β-glucanase or proteinase K initially suggested that some radioactive xyloglucan or glycoprotein may also be formed.

The high molecular weight EDTA extract treated with proteinase K produced an almost complete breakdown of the product with most of the radioactive material running slower than the low molecular weight marker (both on Bio-Gel P10 and Sepharose CL-6B). This could indicate that the proteinase K used in this work gave anomalous results that caused the radioactive material to run slower, possibly due to some interactions with the gel matrix. Alternatively, the proteinase K could be contaminated with β–galactosidase. Thus, this low molecular weight product was collected, concentrated, and run on paper chromatogram in a 10:4:3 (v/v) EPW solvent system [Fig 43]. As compared with silver stained markers the product ran parallel to the marker sugar galactose. Hence the most plausible explanation is that the proteinase K enzyme is contaminated with β–galactosidase activity which was subjected to further investigations.

Conclusion

The conditions for galactan synthase in peas (*Pisum Sativum* L. cv Meteor) were optimised. The best incorporation of UDP-[U-^{14}C]- galactose into the galactan product was at pH5.5 (Mes buffer) with a final concentration of 0.2mmol.l^{-1} UDP-galactose added to the standard incubation and with a pre-centrifugation at 27,000 *g*. Optimal incubation time was 60 minutes. When the different washes of the pellets were analysed, it was found that additional washes could decrease the low molecular weight components containing galactose. However, this was not implemented since these

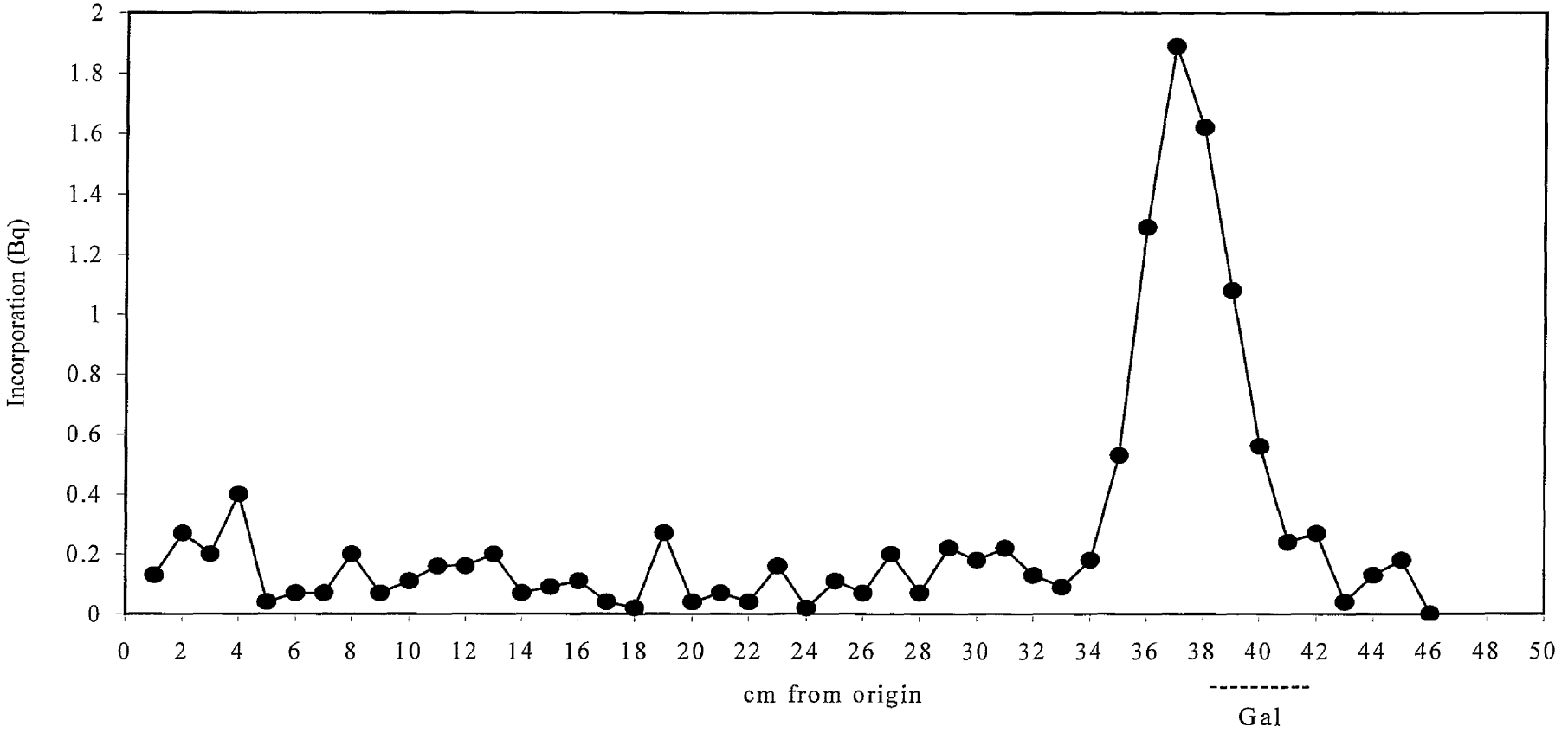

Fig 43. Descending paper chromatography of the low molecular weight fractions from the proteinase K-treated high molecular weight EDTA extract of the particulate enzyme material. The high molecular weight EDTA extract of the particulate enzyme preparation was treated with proteinase K enzyme and run on Bio-Gel P10 column as in Fig 42. The low molecular weight material (in the $CoCl_2$ region) was collected and run on paper chromatography in 10:4:3 ethyl acetate: pyridine: water (v/v) system for 18 hrs. The position for marker sugar galactose (Gal) was determined by silver staining. UDP-galactose ran in fractions 33-36.

washes solubilised some of the needed high molecular weight EDTA extracts. Partial TFA hydrolysis and galactanase time-course experiments produced galactan hydrolysates similar to those obtained by Panayotatos and Villemez (1973). Triton X-100 was found to stimulate incorporation of label into the galactan product. The findings in this chapter show that the product being synthesised was galactan and the conditions for its production were optimised. The galactan product seemed to be attached either to pectin or xyloglucan or both. A protein may also have been involved. More work was next carried out on the attachment of this galactan.

Chapter V

Identification of the Galactan Chain and The Polymers to Which It Is Attached

Chapter V

Introduction

Previous results (such as enzyme breakdown analysis, galactanase time course, and partial TFA hydrolysis) indicated that a galactan was being formed. It was now important to determine whether an endogenous galactanase was still active in the membranes since that would explain why some breakdown occurred in the high molecular weight EDTA extracts incubated in buffer alone. The work described in this chapter was aimed at further identifying the galactan chain and the polymers that might be linked to it. This chapter also addresses the binding properties of the analysed product to the cell wall as well as to the xyloglucan component.

A-Identification of the galactan chain and the polymers to which it is attached

1-Evidence for oligosaccharide series of galactan

The previous chapter has already provided some evidence for the presence of oligosaccharide series resulting from the partial TFA hydrolysis or enzymatic treatment of galactan. But some doubt had arisen from the breakdown of some high molecular weight EDTA extract in the control treatments at a specific pH and temperature to produce intermediate and low molecular weight products. An endogenous galactanase seemed to still be active in the EDTA extracts. This endogenous galactanase enzyme appeared to withstand all the harsh conditions used in the preparation of the particulate enzyme material and the EDTA extract. The particulate enzyme preparation was incubated with UDP-[^{14}C]- galactose for 0 min, 30 min, 60 min, 2 hrs, 18hrs, and 24hrs

and terminated with 90% ethanol. After centrifugation the supernatant was run on paper chromatography in a 10:4:3 (v/v) EPW solvent system [Fig 44]. The galactose marker ran between fractions 26-29. Comparing the results of the chromatography with the work of Panayotatos and Villemez (1973) it was found that that the hexamers, pentamers (Rgal<0.2), and tetramers (Rgal=0.25) were present in all the supernatants obtained from this time-course study. However, they became less abundant with time. The trimer that was present in fractions 9-11 seemed also to decrease with time but at a lower rate. On the other hand, the dimer (galactobiose) in fractions 16-19 and the galactose monomer seemed to increase with time. Thus the galactan product was being degraded with time into its oligosaccharide components. To study if the control of galactanase-treated EDTA extract behaved in a similar fashion, some EDTA extracts were adjusted to the same conditions to test galactanase activity (pH 4.5 with sodium acetate buffer at 40°C) and kept for different time intervals (0, 4, 24, and 31 hours). They were then run on paper chromatography in a 10:4:3 (v/v) EPW system. Compared to the work of Panayotatos and Villemez (1973) it was found that EDTA extracts kept for 0 or 4 hour (at these conditions) have peaks at 1cm (hexamer or pentamer), 6-8cm (tetramer), 10-12cm (trimer), 21-24cm (an unidentified oligomer and probably UDP-galactose), and at 26-29 cm (galactose) [Fig 45]. Extracts kept for 24 or 31 hours had similar peaks but more radioactive material was present in the galactobiose (16-18cm) position. This suggests that the endogenous galactanase could still be active in the EDTA extract and therefore treatments with incubation time 4 hours or less are recommended.

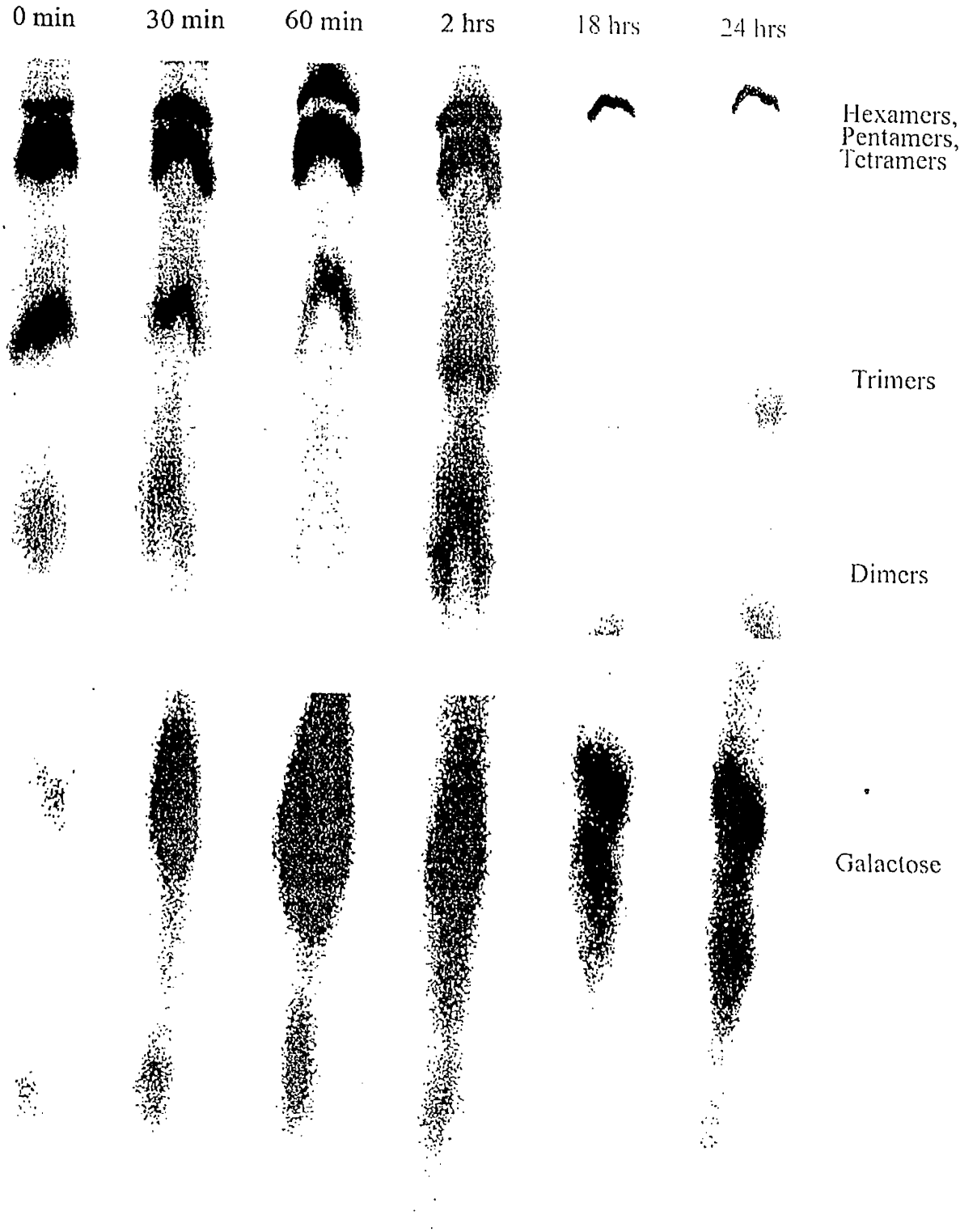

Fig 44. Descending paper chromatography of the 90% ethanol used in terminating the incubations of the particulate enzyme preparations at different time intervals. The particulate enzyme preparation was incubated with UDP-[U-^{14}C]-galactose for 0 min, 30 min, 1 hr, 2 hrs, 18 hrs, and 24 hrs. After centrifugation, the supernatant was run on descending paper chromatography in a 10:4:3 EPW (v/v) system for 18hrs. The position for marker sugars galactose (Gal) was determined by silver staining. The positions of the different oligomers of galactose were determined by comparison with the work of Panayotatos and Villemez (1973). Radioactivity was detected with the help of an IMAGER.

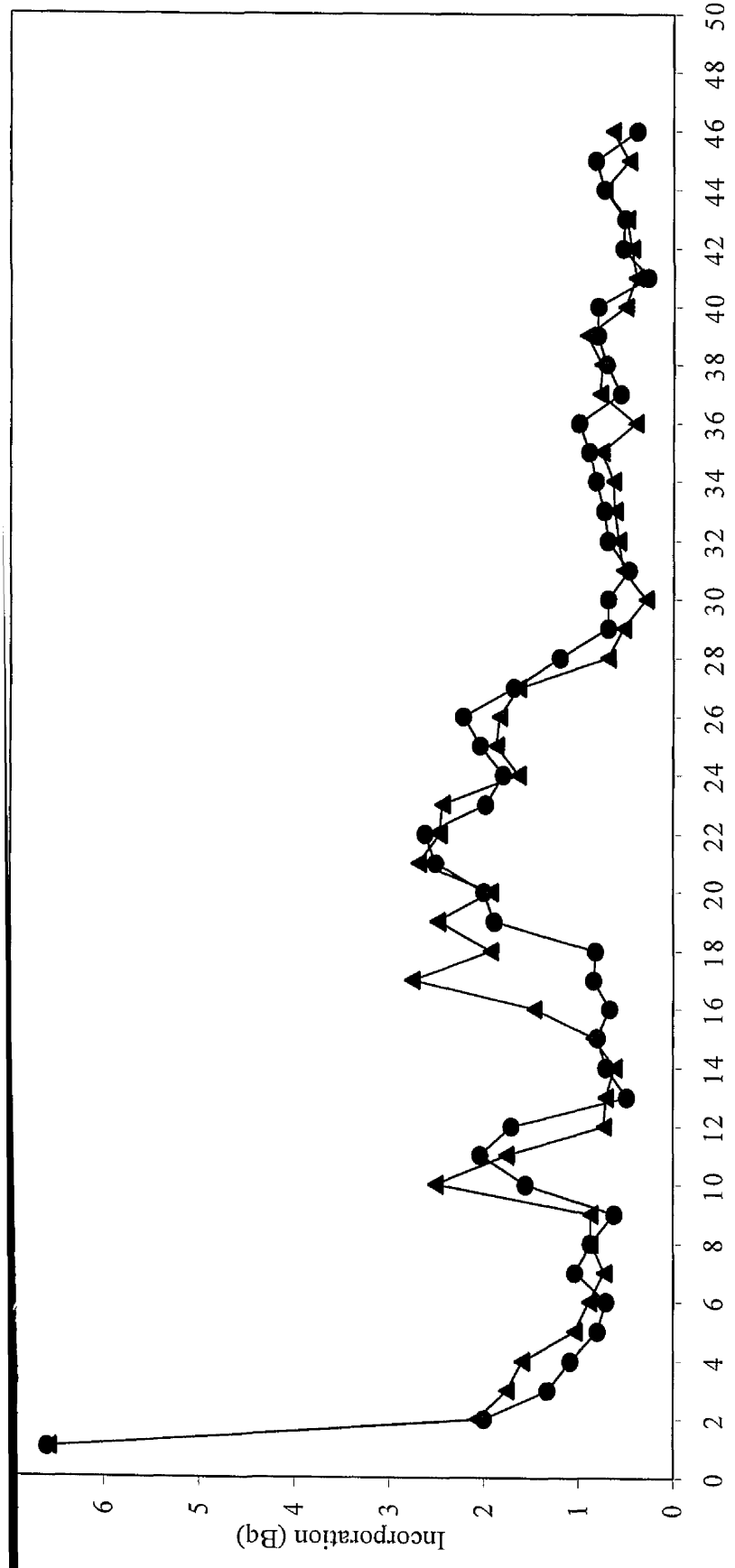

Fig 45. Descending paper chromatography of the EDTA extracts (incubated at specific conditions) of the particulate enzyme preparation. EDTA extracts were adjusted to the conditions optimal for galactanase activity (pH 4.5, 40°C) and kept for different time intervals. These extracts were next run on a descending paper chromatography in a 10:4:3 EPW (v/v) system for 18hrs. The position for marker sugars galactose (Gal) was determined by silver staining. The positions of the different oligomers of galactose were determined by comparison with the work of Panayotatos and Villemez (1973). Circles represent 0 or 4 hr (gave very similar results, shown as one curve) incubations whereas triangles represent 24 or 31 hr incubations (gave very similar results, shown as one curve).

2-Enzyme treatments

a- Comparison of proteinase K and bacterial protease activities

Proteinase K was suspected (from results obtained earlier) to be contaminated with galactosidase activity. The high molecular weight EDTA extracts from particulate enzyme preparations were treated with the bacterial protease and proteinase K (fungal) and the supernatants were run on Bio-Gel P10 [Fig 46]. A shift occurred from high to low molecular weight material in the presence of the bacterial protease. As shown previously the proteinase K gave a product (galactose) that ran slower than the low molecular weight marker ($CoCl_2$). To test if proteinase K is contaminated with galactosidase activity, the latter activity was assayed according to the method of Fry (1988). 0.5ml of 5mmol.l^{-1} para-nitrophenyl-β-D-galactoside (ρNP-galactoside) in buffer (50mmol.l^{-1} acetic acid, pH 4.7) was incubated with tested enzyme (0.5ml) for certain time intervals. The incubations were terminated with 1mol.l^{-1} sodium carbonate. The solution was then assayed spectrophotometrically at wavelength 400nm to check for the ρNP that results from the hydrolysis of ρNP-galactoside and that has a yellow colour in alkaline medium [Fig 47]. This indicates that proteinase K is contaminated with β-galactosidase activity.

The high molecular weight EDTA extract, treated with bacterial protease was studied further by running it on Sepharose CL-6B to test for the existence of intermediate peaks as those reported by Crosthwaite *et al.* (1994). This was not the case as evident in Fig 48. However, the possibility that the product formed may be linked to a protein cannot be ruled out. The degraded product might still be of high molecular weight material and hence eluting in the void volume with the high molecular weight marker (Blue Dextran).

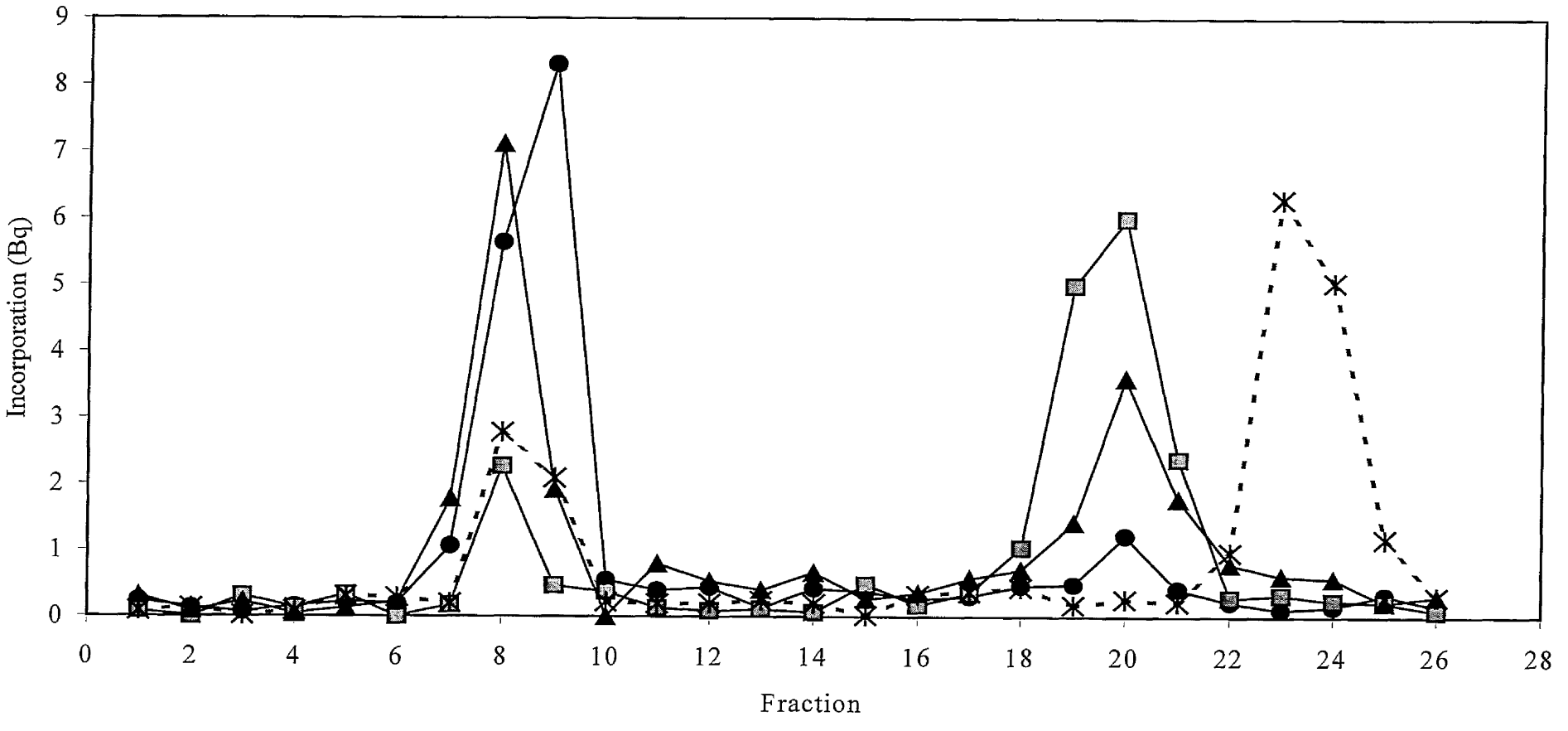

Fig 46. Gel filtration on Bio-Gel P10 column after treating the high molecular weight EDTA extract of the particulate enzyme material with different enzymes. The same treatment was used for control (circles) but with no enzyme. The extracts were treated with galactanase (squares), bacterial protease (triangles), or fungal proteinase K (crosses). The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 18-23.

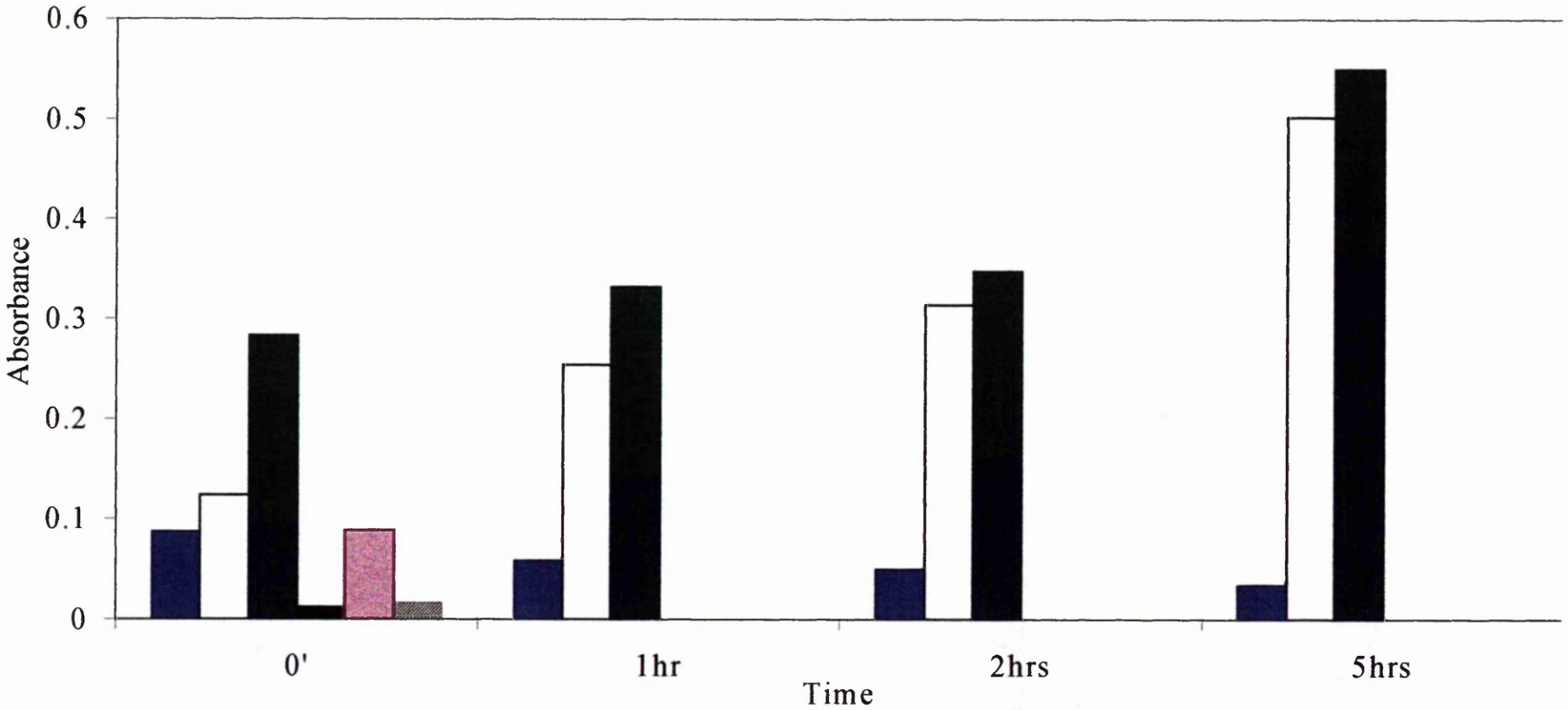

Fig 47. Test for contamination of proteinase K (fungal) with β-galactosidase activity according to the method of Fry (1988). Time-course treatments of tested enzyme with substrate (para-nitrophenyl-β-D-galactopyranoside in buffer). The solution is assayed spectrophotometrically at 400nm to check for change in colour. Different controls were performed: controls with substrate but no enzymes (blue bars), controls with no enzymes and no substrate (black bars), controls with proteinase K but no substrate (purple bars), and controls with galactosidase but no substrate (bars with diagonal lines). Proteinase K treatments (empty bars) and galactosidase treatments (green bars) are also shown.

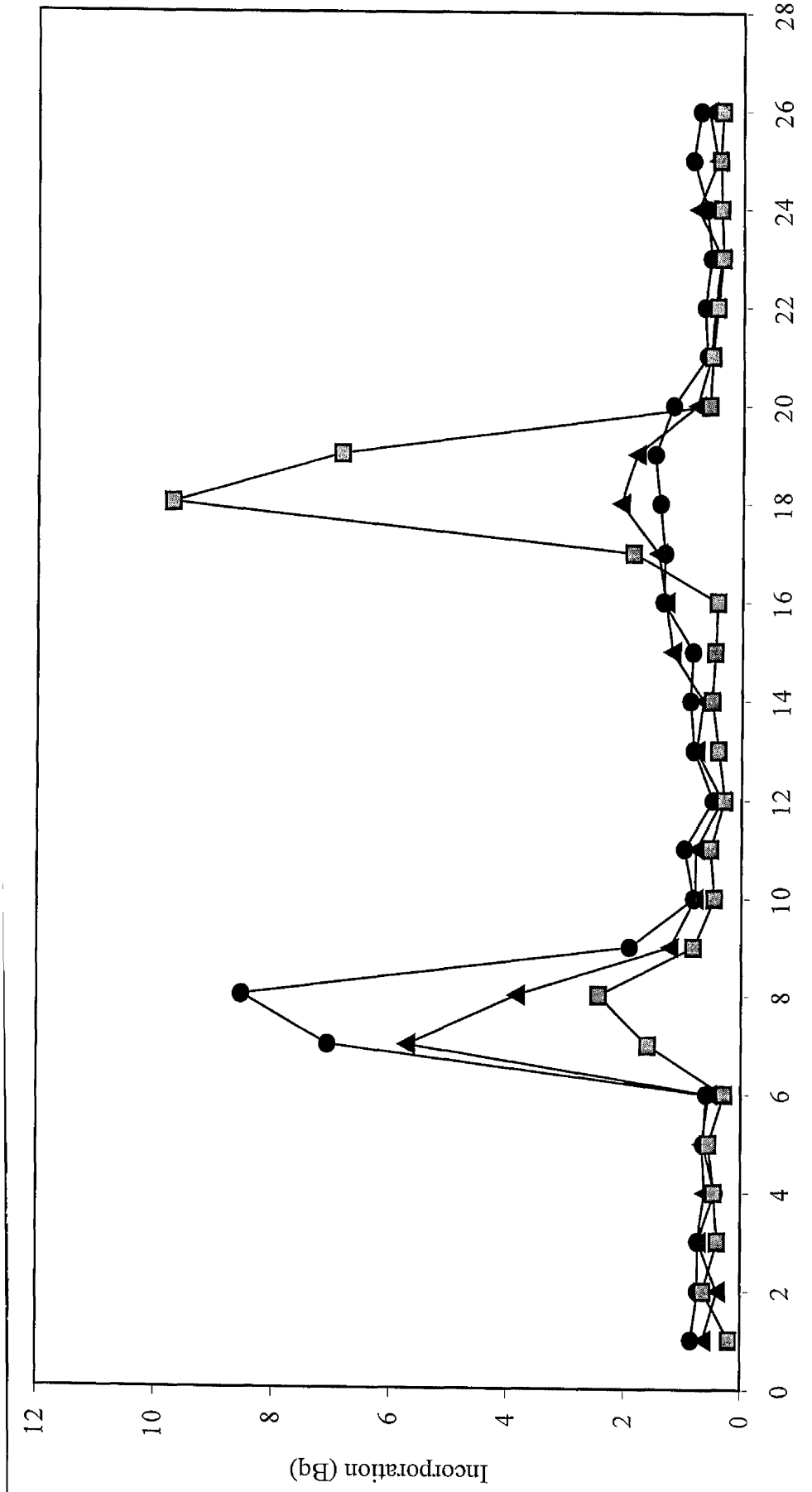

Fig 48. Gel filtration on Sepharose CL-6B column after treating the high molecular weight EDTA extract of the particulate enzyme preparation with different enzymes. The same treatment was used for control but with no enzyme. The extracts were treated with galactanase (squares), bacterial protease (triangles), or as controls (circles). The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 17-21.

b- Chromatography using Bio-Gel P10 columns

The high molecular weight EDTA extract was further analysed by incubating it for half an hour with different combinations of enzymes [Fig 49 and Fig 50]. Upon treatment with polygalacturonase (PG) or pectin lyase (PL) a shift in the peak occurred suggesting the attachment of the galactan product to pectin. The endo-1,4-β-glucanase degraded the product as evident from the presence of an intermediate molecular weight peak. This indicates that the radioactive product most probably consisted of XG attached to [^{14}C]-galactan rather than of [^{14}C]-galactose attached to XG; otherwise endo-1,4-β-glucanase would have resulted in the degradation of [^{14}C-galactose]-XG to low molecular weight material (for example: [^{14}C]-nonasaccharide). Some [^{14}C]-galactan might be attached to XG, others to pectin, and a complex of all three together might also be a possibility. The fact that the action of PG and endo-1,4-β-glucanase enzymes was not additive may indicate that they are acting on the same complex. Treatment with rhamnogalacturonase B also produced a shift from high molecular weight into intermediate molecular region suggesting the attachment of the 1,4-β-galactan to an RGI-PGA backbone [Fig 51]. A difference in the intermediate peaks produced by rhamnogalacturonase B or by polygalacturonase enzyme exists [Fig 49, Fig 51]. The intermediate peak produced by polygalacturonase enzyme was broad and flat indicating the production of oligomers of different sizes attached to the [^{14}C]-galactan [Fig 49]. The intermediate peak produced by rhamnogalacturonase B degradation gave a single intermediate peak of a greater Kav than peaks obtained by polygalacturonase degradation. No change in elution behaviour was detected when the radioactive polymer was run through Bio-Gel P10 in the presence of 8mol.l^{-1} urea,

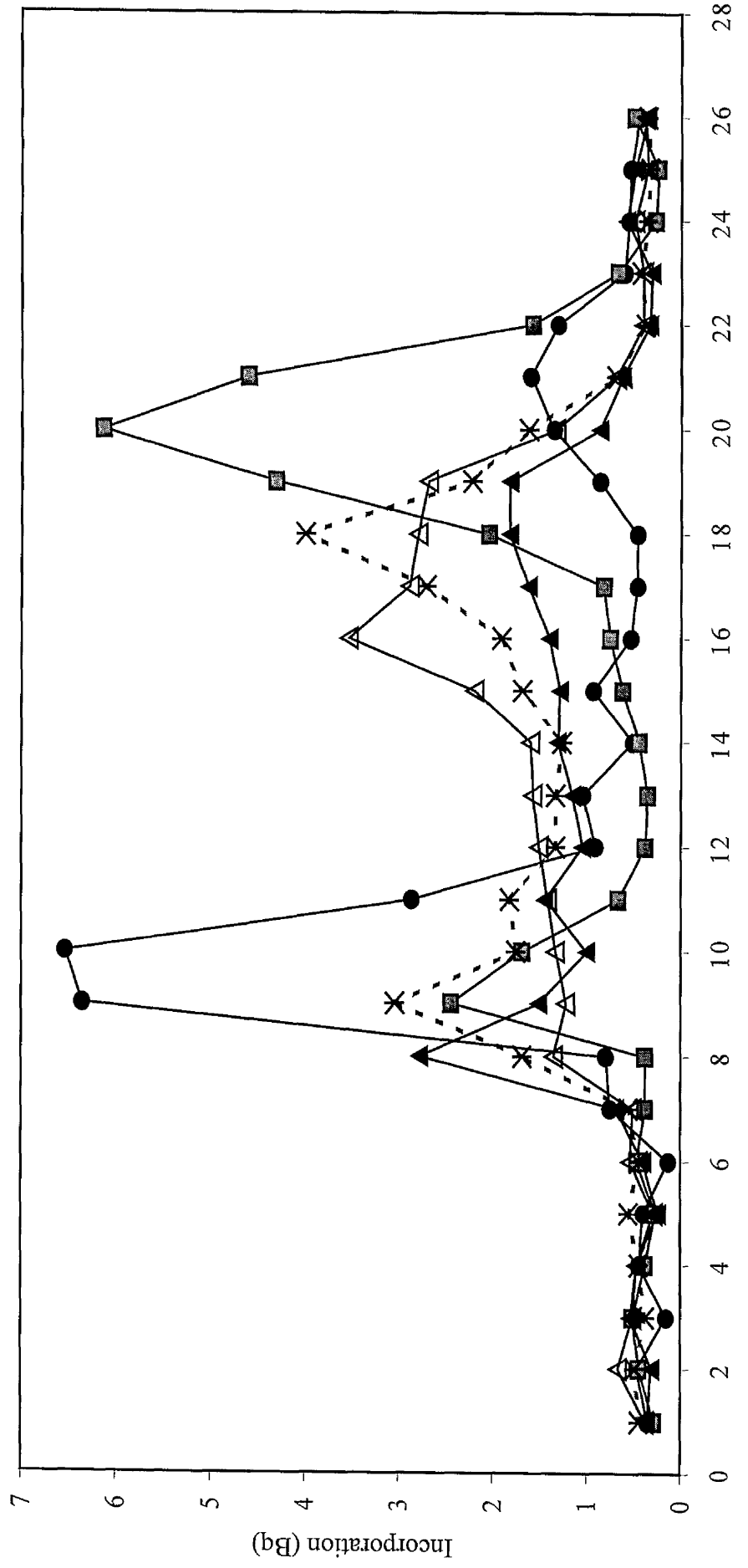

Fig 49. Gel filtration on Bio-Gel P10 column after treating the high molecular weight EDTA extract of the particulate enzyme preparation with different enzymes for 30min. The same treatment was used for control but with no enzyme. The extracts were treated with endo-1,4-β-galactanase (squares), endo-1,4-β-glucanase (open triangles), polygalacturonase (closed triangles), polygalacturonase + endoglucanase (crosses), or as controls (circles). The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 19-22.

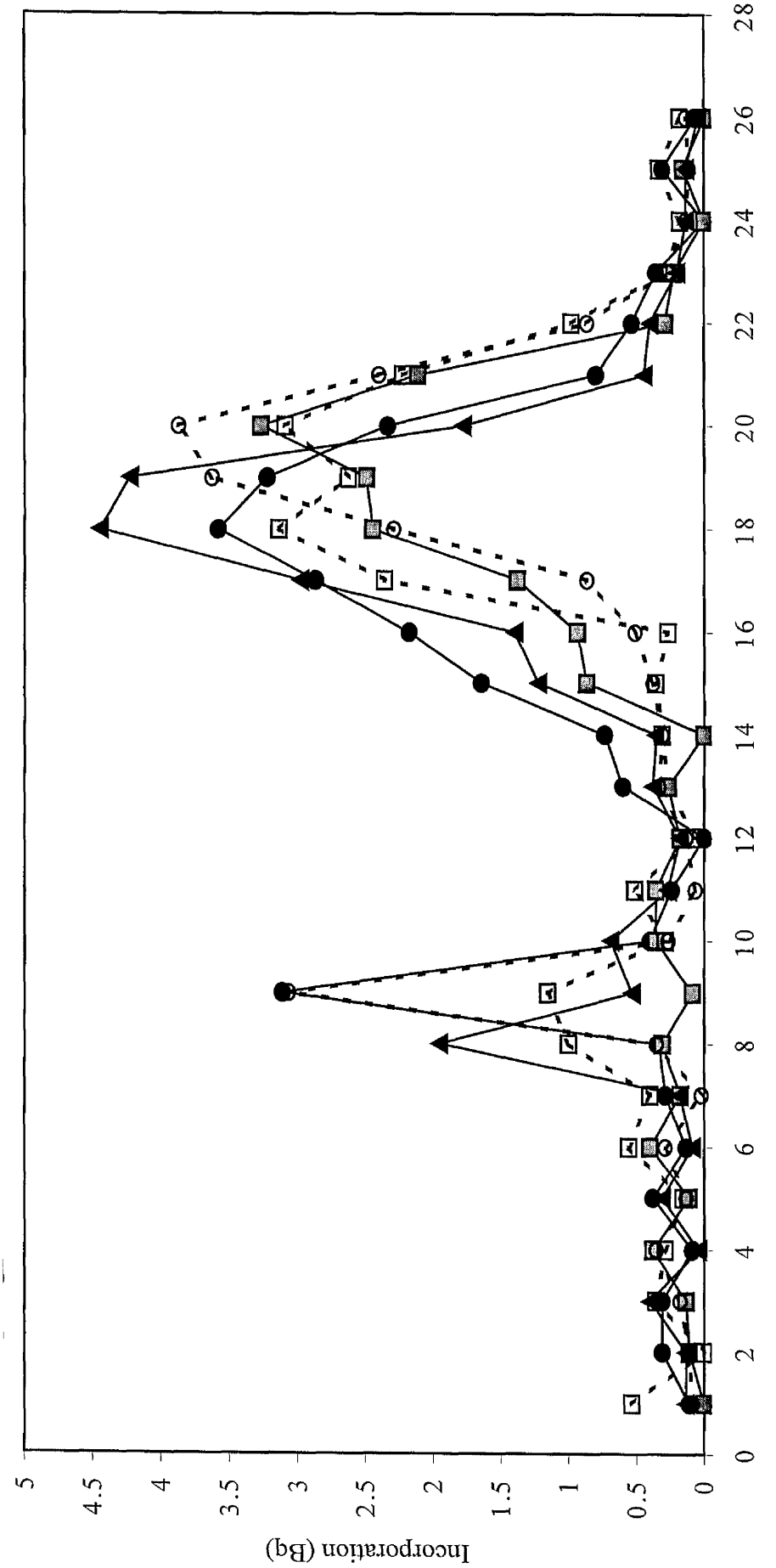

Fig 50. Gel filtration on Bio-Gel P10 column after treating the high molecular weight EDTA extract of the particulate enzyme preparation with different enzymes (as Fig 49) to compare action of polygalacturonase and pectin lyase. The extracts were treated with polygalacturonase (closed circles), pectin lyase (closed squares), endo-1,4-β-glucanase (triangles), polygalacturonase + endoglucanase (open circles), or with pectin lyase + endoglucanase (open squares). The Blue Dextran marker ran between fractions 8-12, while the $CoCl_2$ marker ran between fractions 18-23.

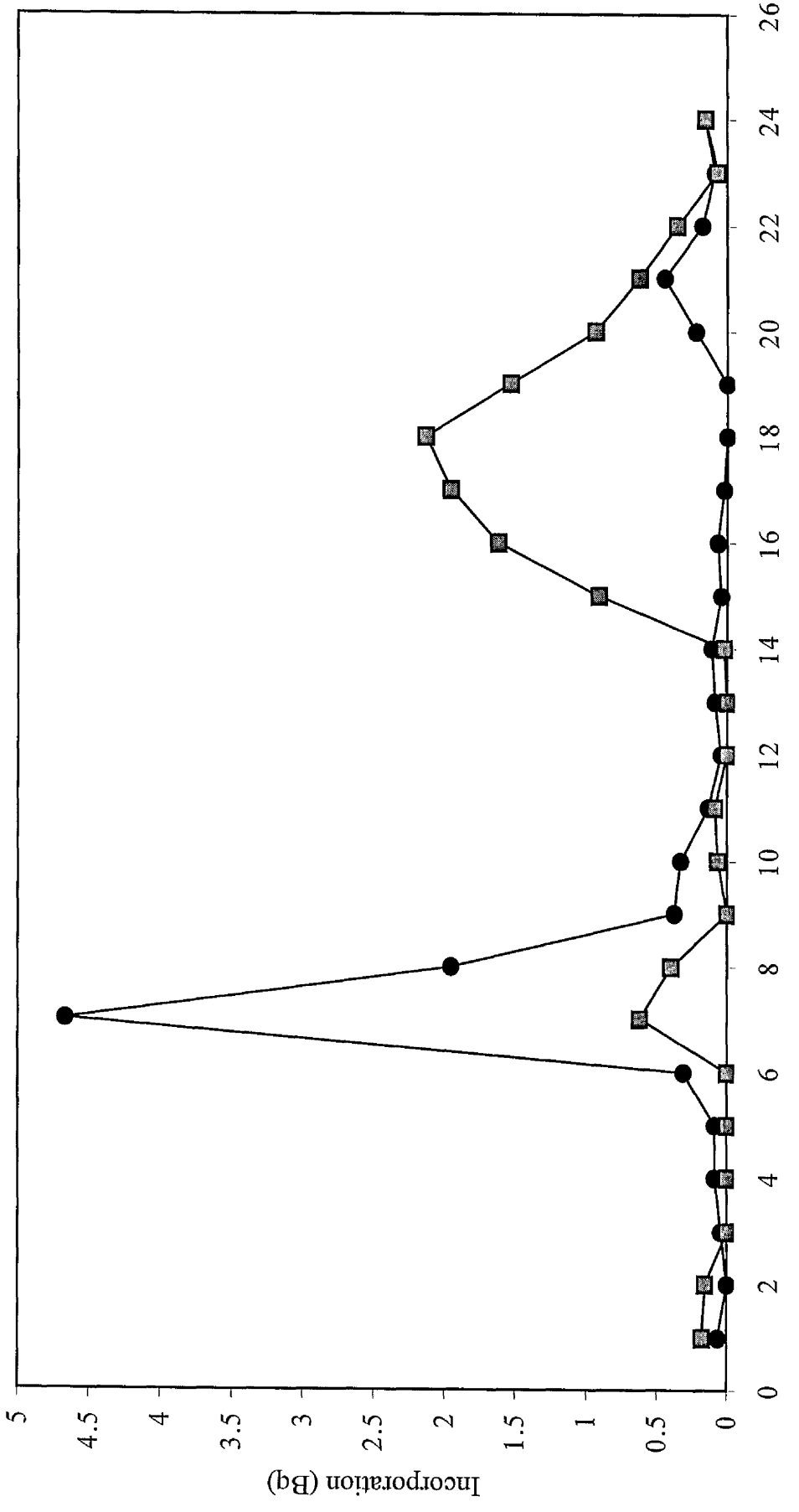

Fig 51. Gel filtration on Bio-Gel P10 column after treating the high molecular weight EDTA extract of the particulate enzyme preparation with rhamnogalacturonase (B). The same treatment was used for control (represented by circles) but with no enzyme. Rhamnogalacturonase B treated extract is represented by squares. The Blue Dextran marker ran between fractions 6-8, while the $CoCl_2$ marker ran between fractions 19-22.

indicating that the components of the polymer were held together by covalent bonds [Fig 52].

All this supported the possibility that we are dealing with complex of galactan, PGA, RGI, and xyloglucan. Yet, more work was necessary to understand how these complexes are attached.

c- Anion-exchange chromatography

The high molecular weight EDTA extract was run on EconoPac–High Q cartidge from Bio-Rad. Some of this material (15-30%) was eluted in the neutral region whereas the majority (70-80%) remained attached to the anion column and was eluted with 0.3 $mol.l^{-1}$ NaCl / 0.2M Tris pH 7.8 buffer which indicated that it was partly acidic [Fig 53]. Galactan is neutral, thus the material eluted in the acidic medium was galactan attached to pectin or to another polymer. The radioactive material that did not bind to the column (that is the material eluted in the buffer with $0mol.l^{-1}$ NaCl) was concentrated and passed through a Bio-Gel P10 column [Fig 54]. This material was eluted in the void volume confirming that it was polymeric. Hence it was probably not free galactan, but attached to either XG or to highly methylated pectin or to a rhamnogalacturonan I with a low charge density.

In the presence of $8mol.l^{-1}$ urea, an even greater portion (90%) of the polymer was found to bind to the ion exchange and most of it required buffer containing $0.5mol.l^{-1}$ NaCl for elution [Fig 55]. This increased binding in the presence of urea may either be due to decrease in water content or due to the removal of a neutral non-covalently bound protein. No significant change in binding in the presence of urea occurred after treatment of the radioactive polymer with bacterial protease, indicating

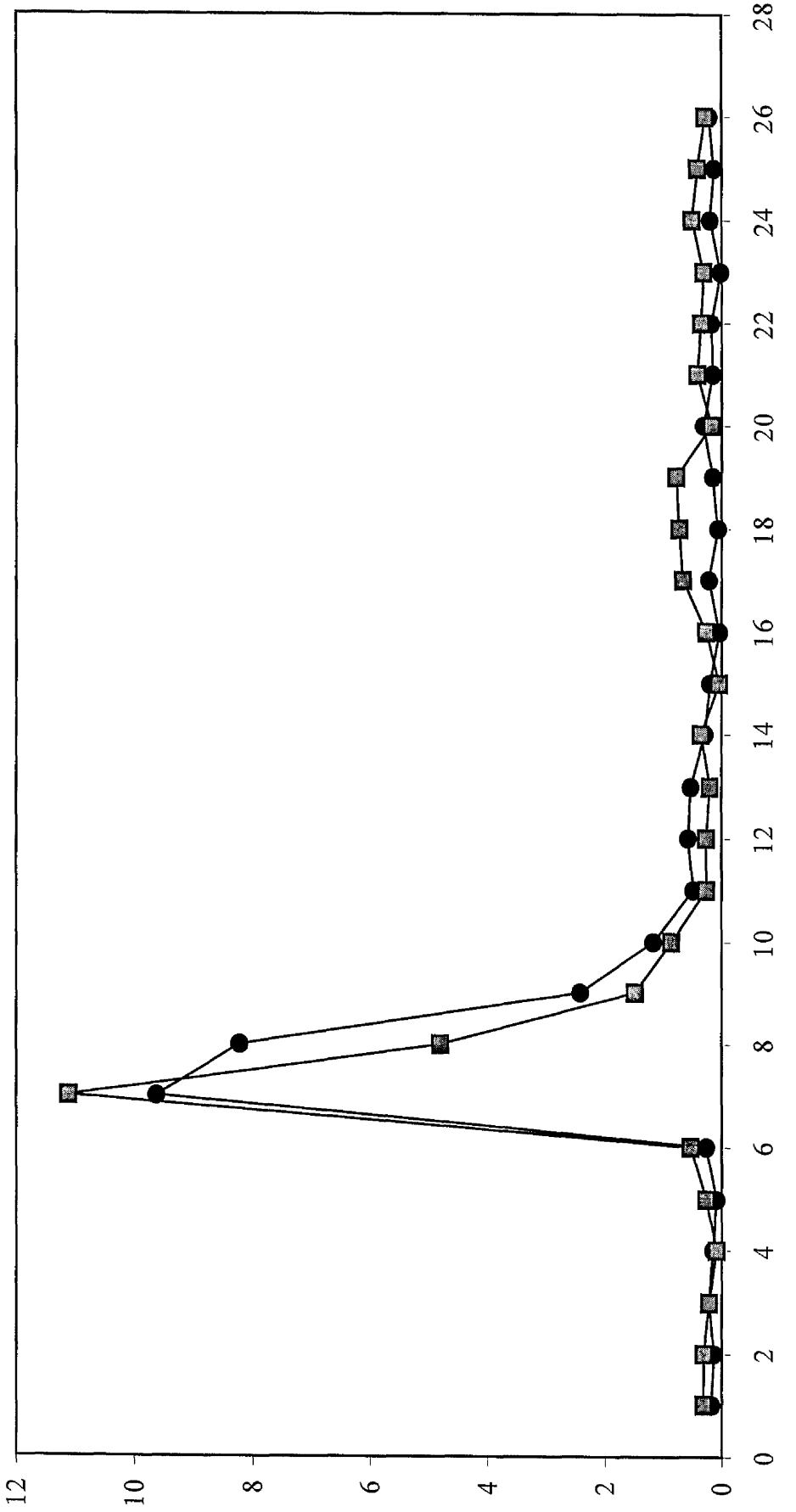

Fig 52. Gel filtration on Bio-Gel P10 column of the high molecular weight EDTA extract of the particulate enzyme preparation with or without (control) 8mol.l^{-1} urea. High molecular weight EDTA extracts with urea are represented by squares, whereas controls are represented by circles. The Blue Dextran marker ran between fractions 7-9, while the $CoCl_2$ marker ran between fractions 18-22.

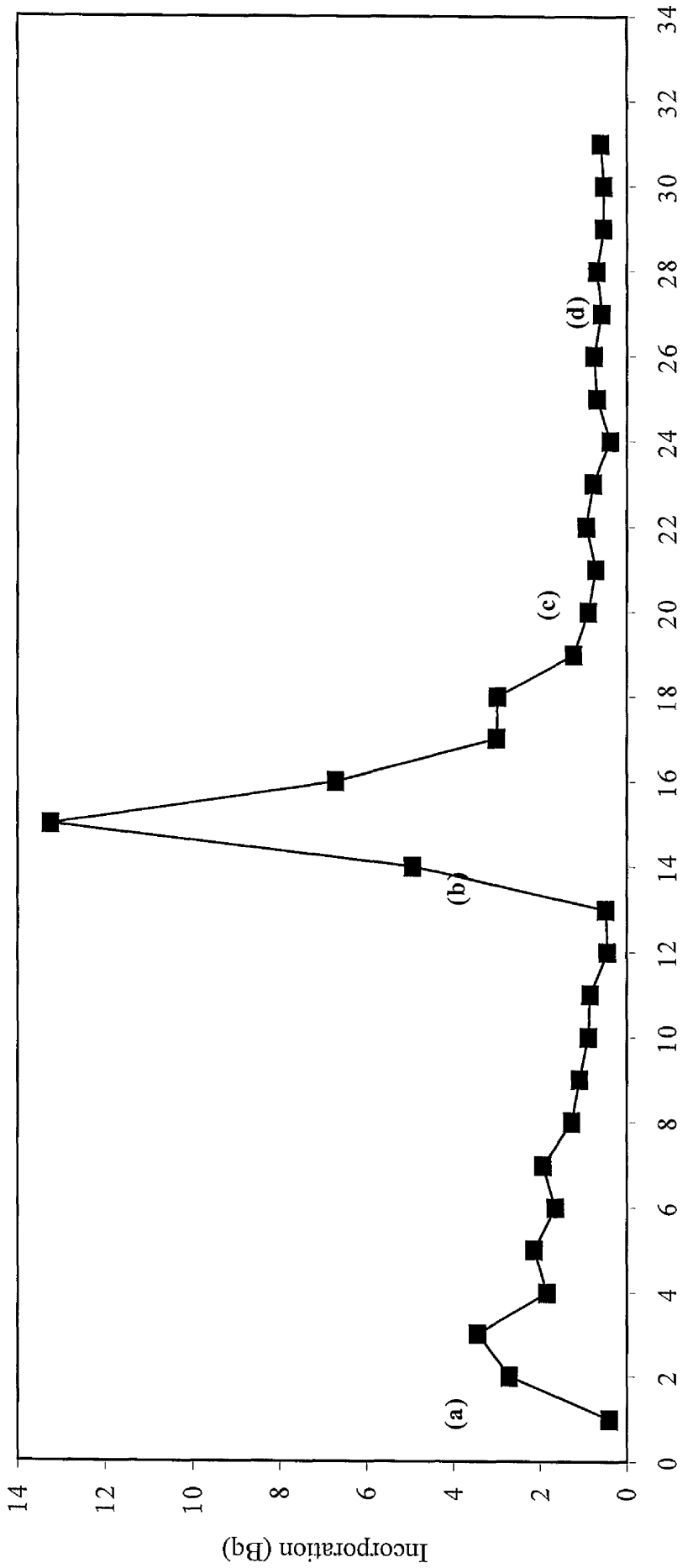

Fig 53. Fractionation by anion exchange of the high molecular weight EDTA extract of the particulate enzyme preparation. The high molecular weight EDTA extract was run on an EconoPaq High Q cartidge and was eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer.

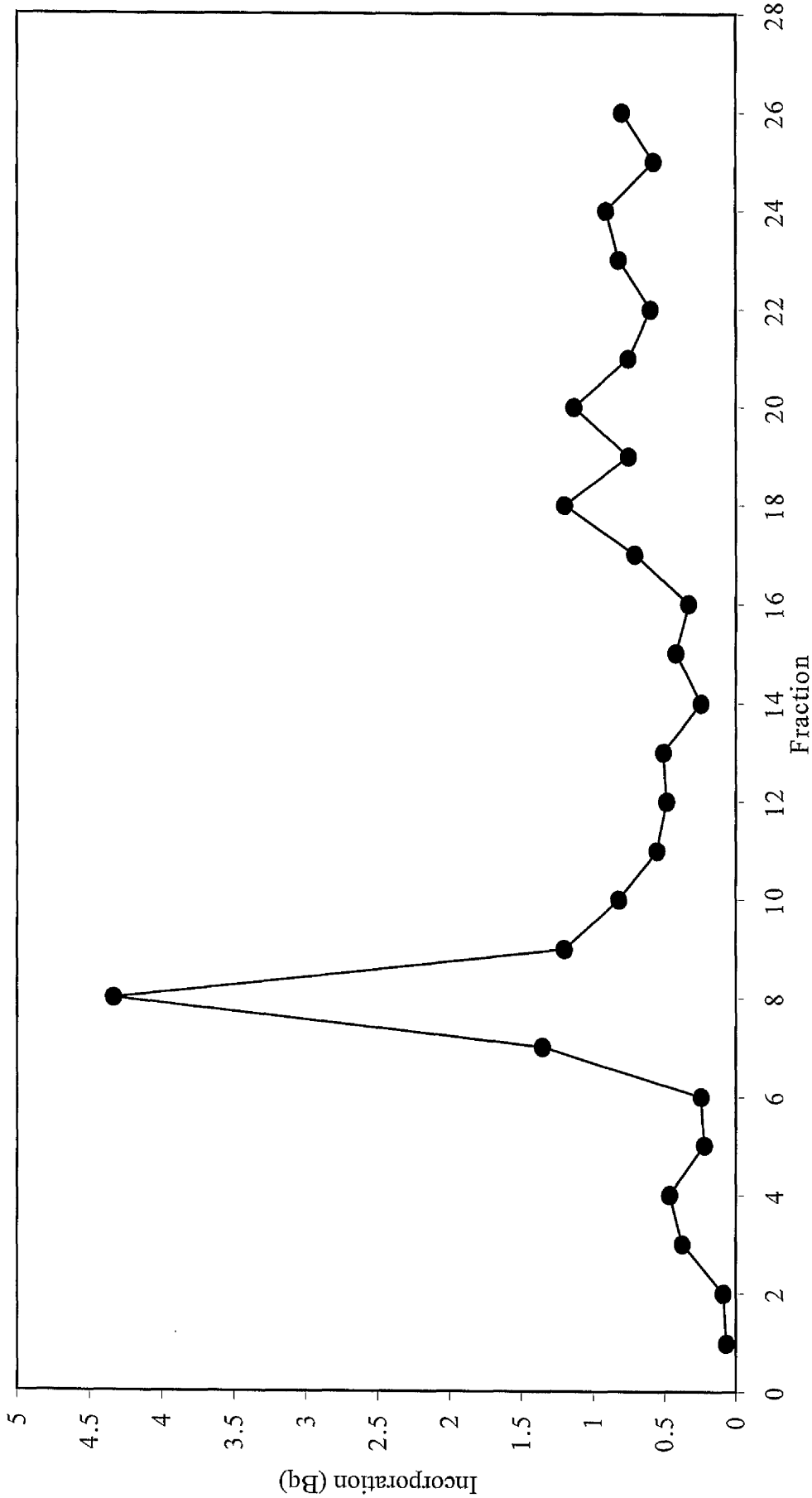

Fig 54. Gel filtration on Bio-Gel P10 column of the material that did not bind to the anion exchange column.
The high molecular weight EDTA extract of the particulate enzyme preparation was run on anion exchange column as in Fig 53 and the material eluted in the 0mol.l^{-1} NaCl buffer was concentrated and run on a Bio-Gel P10 column. The Blue Dextran marker ran between fractions 7-10, while the $CoCl_2$ marker ran between fractions 20-24.

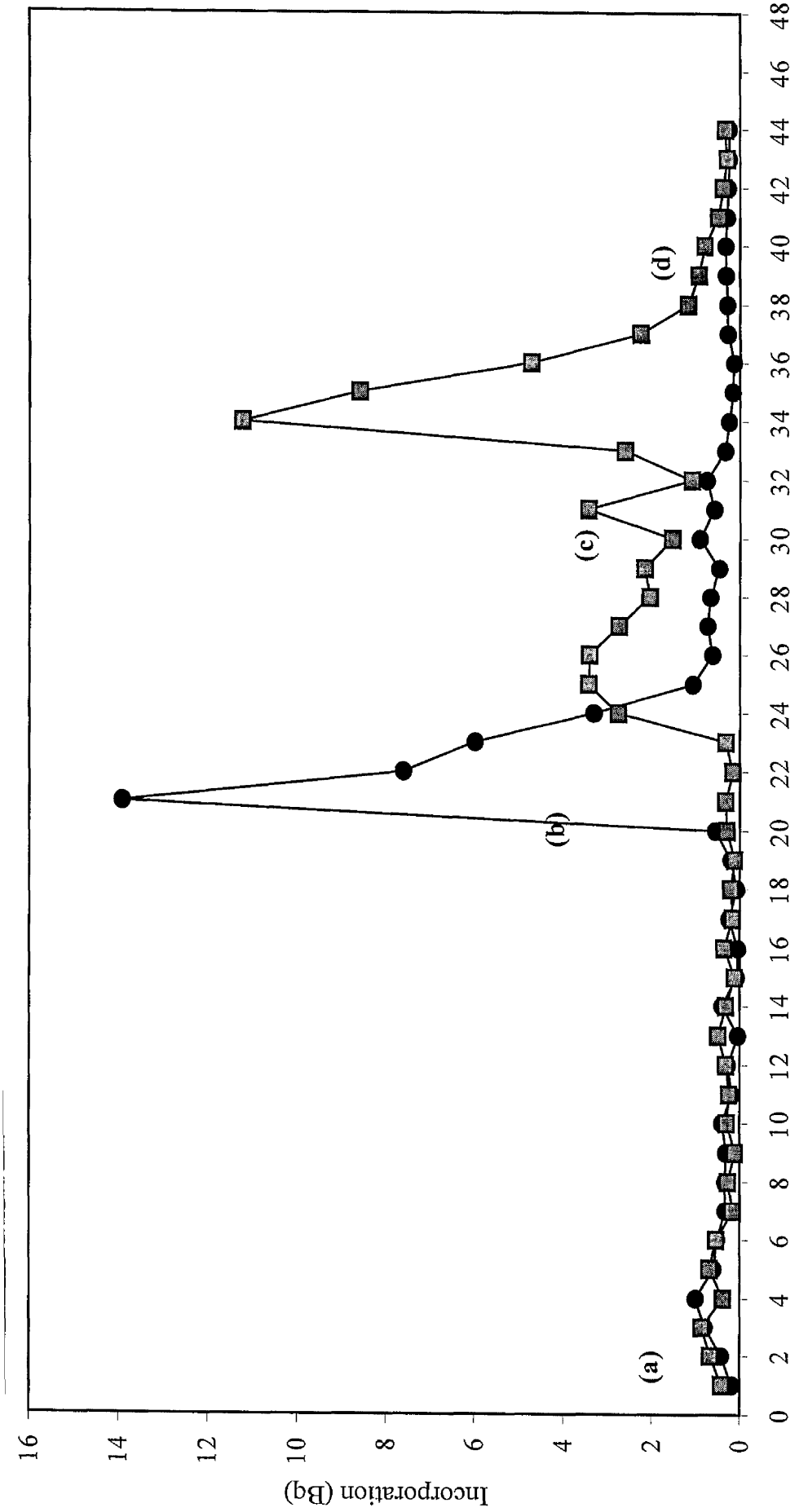

Fig 55. Fractionation by anion exchange of the high molecular weight EDTA extract of the particulate enzyme preparation in the presence or absence of 8 mol.l^{-1} urea. The high molecular weight EDTA extract was run on an EconoPaq High Q cartidge and was eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer. Squares represent the elution in the presence of urea, whereas circles represent elution without urea.

that the binding was not due to an acidic protein [Fig 56]. No change was also observed in binding after incubation with pectin methyl esterase even in the presence of 50mmol.l^{-1} EDTA. EDTA was added to remove the possibility of a cation binding to the negative portions of the product and thus allowing it to elute with the neutral portion [Fig 57].

Treatment of the high molecular EDTA extract with polygalacturonase or endo-1,4-β-glucanase [Fig 58] under 8mol.l^{-1} urea conditions revealed a shift from the acidic to the neutral fraction. Treatment of the high molecular weight EDTA with endo-1,4-β-glucanase (without 8mol.l^{-1} urea conditions) and then running it on anion-exchange column [Fig 59] resulted in a similar shift from acidic to neutral fractions. Further investigations were done by incubating the particulate membrane with UDP-[U-^{14}C]-xylose or GDP-[U-^{14}C]-fucose and applying their high molecular weight EDTA extracts to the anion exchange column. Significant amounts of radioactivity from both preparations were found to bind to the anion exchange column. For both [^{14}C]-xylose [Fig 60] and [^{14}C]-fucose [Fig 61], the proportion of bound radioactivity decreased upon pre-treatment with endo-1,4-β-glucanase, by 44% and 33% respectively providing more evidence for the attachment of xyloglucan to an anionic polymer, probably pectin.

3-Purity of endo-1,4-β-glucanase

Selective enzyme degradation and anion exchange chromatography gave enough evidence that the pectic galactan polymer was also attached to xyloglucan provided that the endo-1,4-β-glucanase enzyme utilised was not contaminated with some pectin degrading enzyme such as polygalacturonase or pectin lyase. Viscometric experiments were performed to clarify this matter. Xyloglucan (1%) was found to be too viscous to

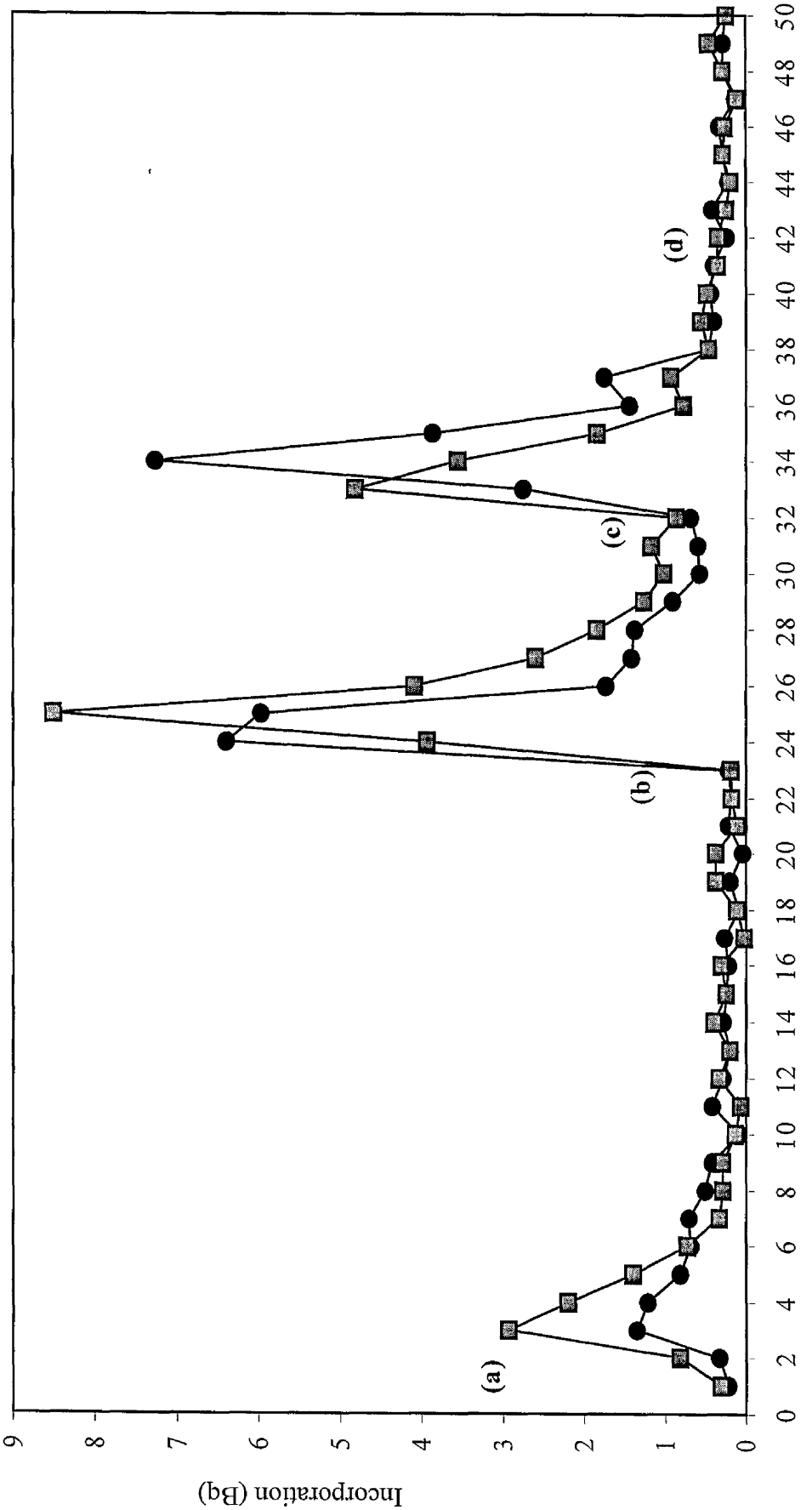

Fig 56. Fractionation by anion exchange of the protease-treated high molecular weight EDTA extract of the particulate enzyme preparation in the presence of 8 mol.l^{-1} urea. The treated high molecular weight EDTA extract was run on an EconoPaq High Q cartidge and was eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer. The control consists of untreated extract (represented by circles) run in the presence of urea. The protease-treated extract is represented by squares.

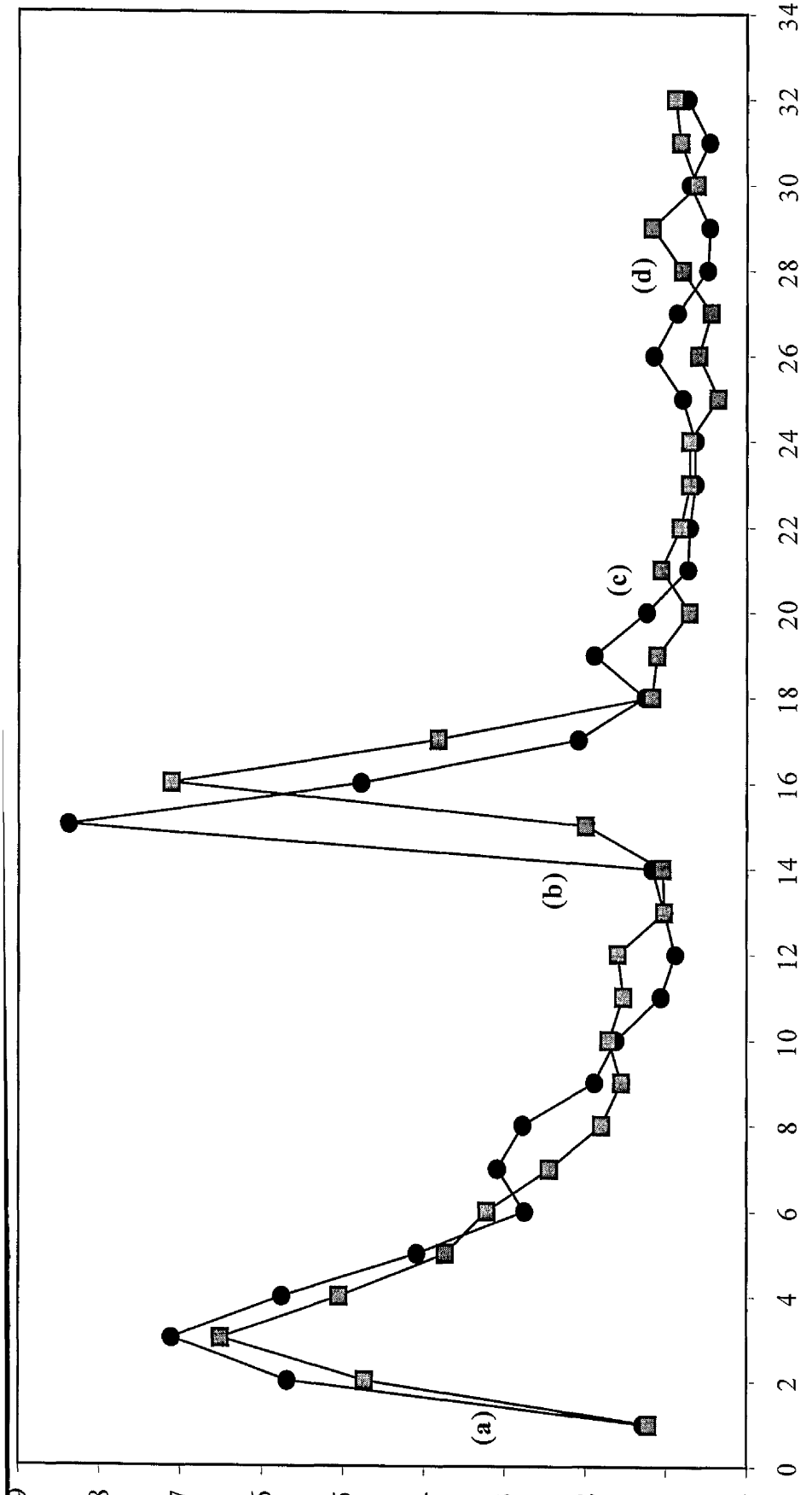

Fig 57. Fractionation by anion exchange of the pectin methyl esterase-treated high molecular weight EDTA extract of the particulate enzyme preparation in the presence of 50 mmol.l^{-1} EDTA. The treated high molecular weight EDTA extract was run on an EconoPaq High Q cartidge and was eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer. The control consists of untreated extract (represented by circles) run in the presence of EDTA. The pectin methyl esterase-treated extract is represented by squares.

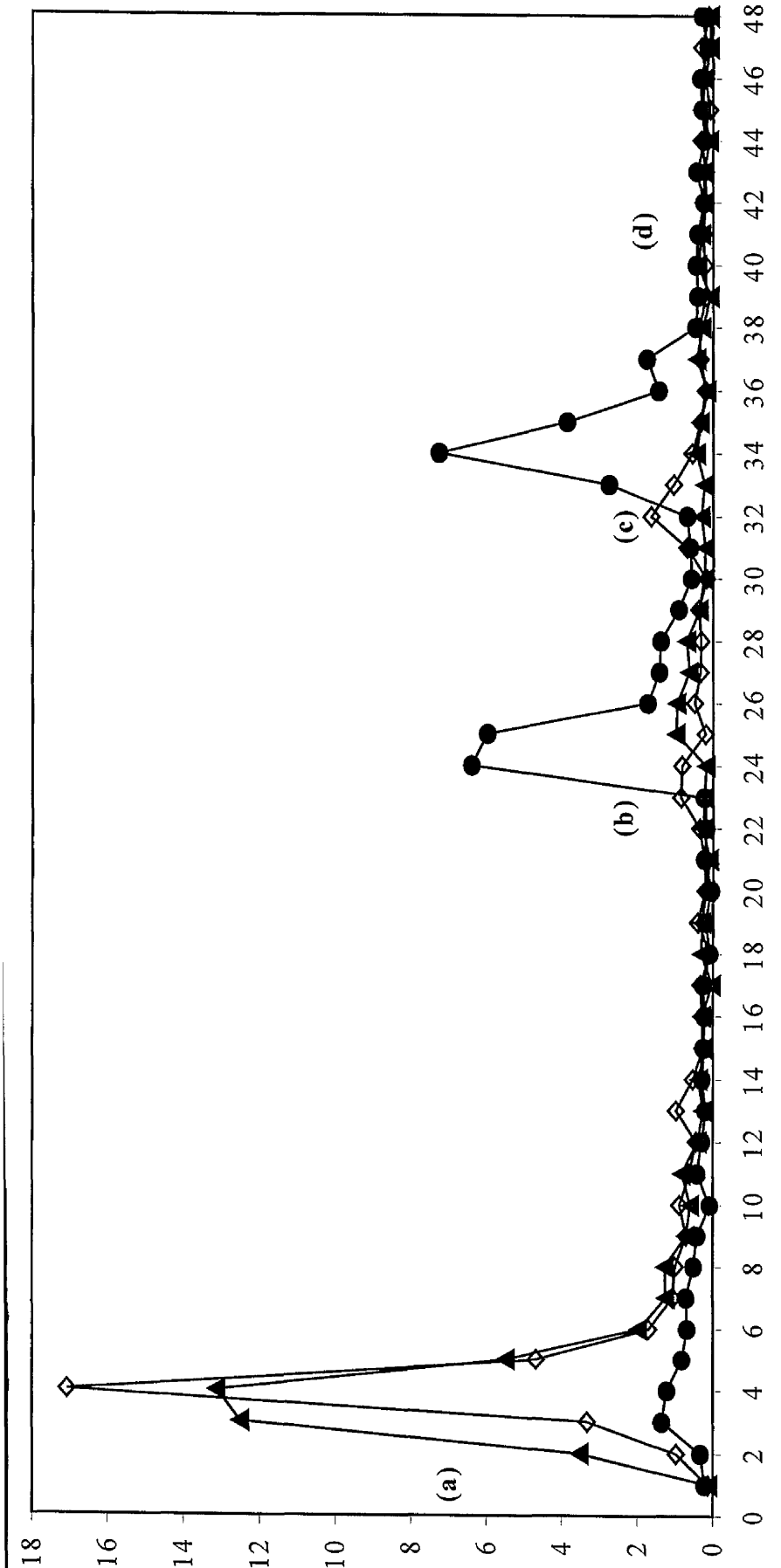

Fig 58 . Fractionation by anion exchange of the endo-1,4-β-glucanase or polygalacturonase-treated high molecular weight EDTA extract of the particulate enzyme preparation in the presence of 8 mol.l^{-1} urea. The treated high molecular weight EDTA extracts were run on an EconoPaq High Q cartidge and were eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer. The control consists of untreated extract (represented by circles) run in the presence of urea. The endo-1,4-β-glucanase are represented by diamonds and the polygalacturonase-treated extracts are represented by triangles.

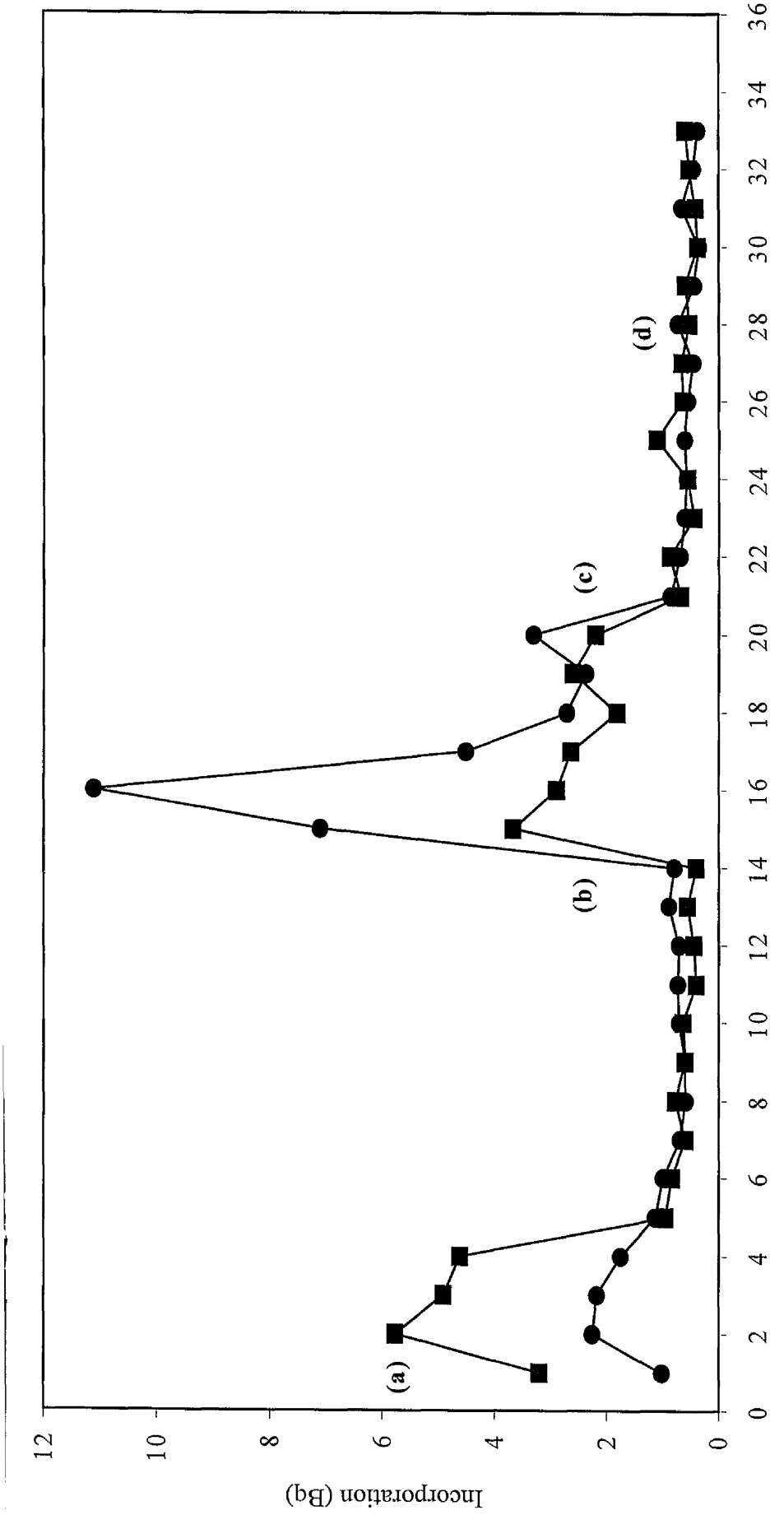

Fig 59. Fractionation by anion exchange of the endo-1,4-β-glucanase treated high molecular weight EDTA extract of the particulate enzyme preparation (in the absence of 8 mol.l^{-1} urea). The treated high molecular weight EDTA extracts were run on an EconoPaq High Q cartidge and were eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer. The same treatment was used for control (represented by circles) but with no enzyme. The endo-1,4-β-glucanase treated extract is represented by squares.

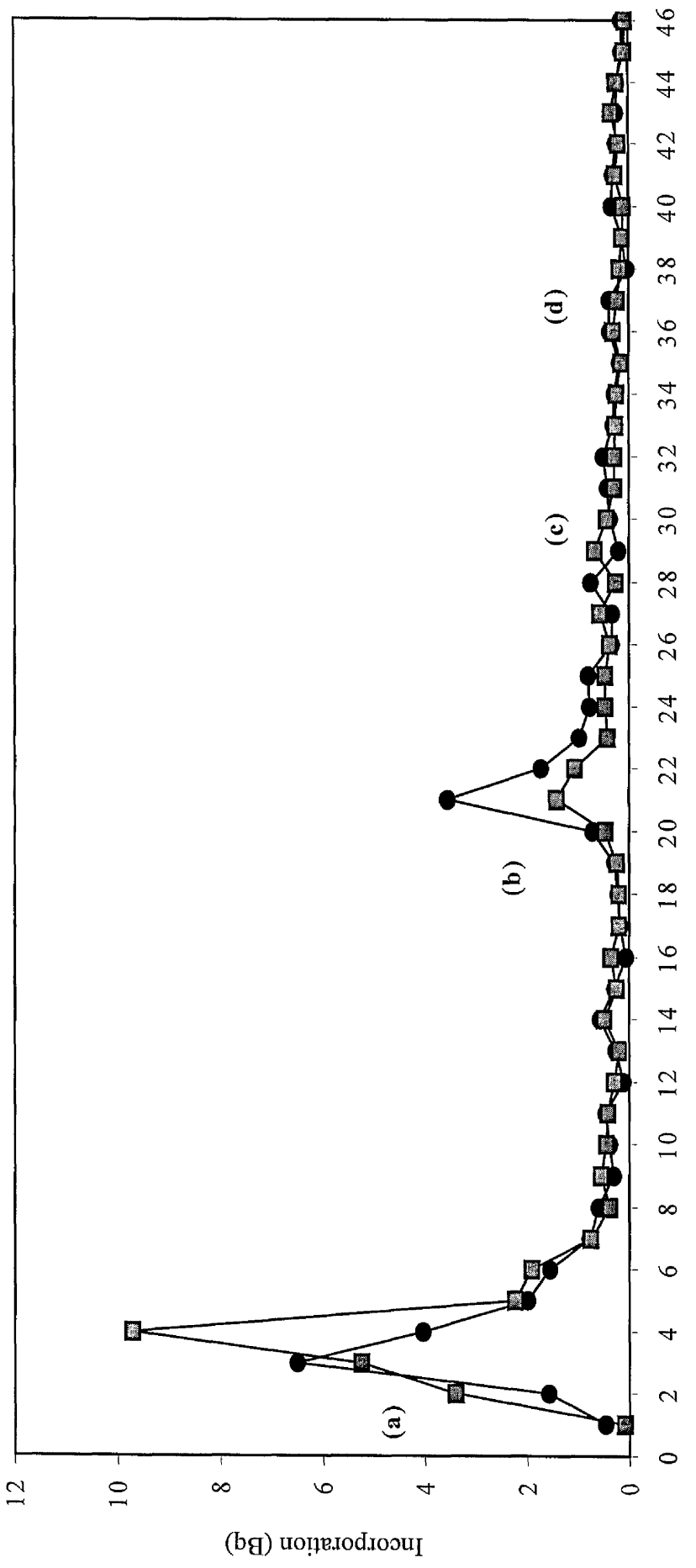

Fig 60. Fractionation by anion exchange of the treated-high molecular weight EDTA extract of the particulate membrane preparation incubated with UDP-[^{14}C]-xylose. These extracts were treated with endo-1,4-β-glucanase (represented by squares) or as control (represented by circles) before they were fractionated by anion exchange (in the presence of 8 mol.l^{-1} urea). The treated extracts were run on an EconoPaq High Q cartidge and were eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer. The same treatment was used for control but with no enzyme.

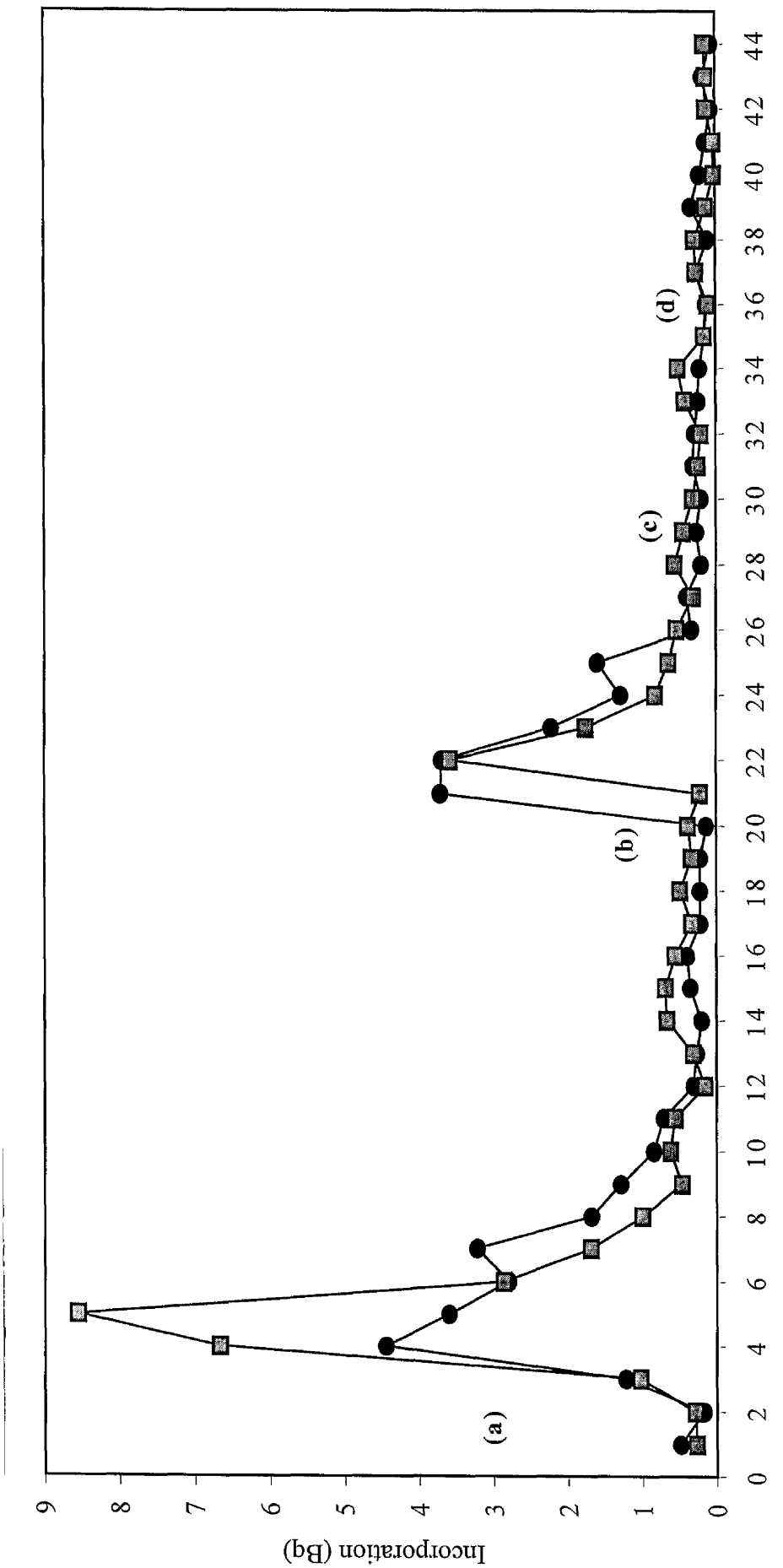

Fig 61. Fractionation by anion exchange of the treated-high molecular weight EDTA extract of the particulate membrane preparation incubated with GDP-[^{14}C]-fucose. These extracts were treated with endo-1,4-β-glucanase (represented by squares) or as control (represented by circles) before they were fractionated by anion exchange (in the presence of 8 mol.l^{-1} urea). The treated extracts were run on an EconoPaq High Q cartidge and were eluted successively with (a) 0, (b) 0.3, (c) 0.5, and (d) 1mol.l^{-1} NaCl in 0.2 mol.l^{-1} Tris (pH 7.8) buffer. The same treatment was used for control but with no enzyme.

run in the viscometer and therefore 0.5% xyloglucan and pectin where compared. In some experiments 1% pectin was used to allow more time for the flow from the viscometer [Table 15, Table 16]. It is evident from these Tables that the endo-1,4-β-glucanase activity was free from any polygalacturonase or pectin lyase activity.

B-Binding to the cell wall and to xyloglucan

Binding of the high molecular weight EDTA extract from the particulate enzyme preparation to xyloglucan and to cell wall preparations (see Materials and Method) was found to be pH-dependent. Binding to the cell wall was found to be highest at pH 3 (about 35%) and decreased gradually reaching to about 5% at pH 5 [Fig 62]. Binding of the high molecular weight EDTA to xyloglucan dropped from 83% at pH 3 to 49% at pH 6 [Fig 63]. Thus it appeared that the product was binding mainly to xyloglucan in the cell wall in a pH dependent manner. To further understand which part of our complex polymer is responsible for this binding, the high molecular weight EDTA extract was treated with galactanase and other enzymes prior to binding to xyloglucan [Table 17]. Galactanase and polygalacturonase enzymes caused the highest decrease in binding. Endo-1,4-β-glucanase and pectin lyase also caused a large decrease in binding. Only the bacterial protease did not show any decrease in binding. Therefore, it could be postulated that all the components of the galactan-pectin-xyloglucan complex are important in helping in the binding to the cell wall, at low pH. To test the importance of the galactan component of this complex, radioactive pectin was prepared with UDP-[^{14}C]-glucuronic acid and binding to xyloglucan was performed after treatment with galactanase. This resulted in a 60% drop in the binding [Fig 64], suggesting that the galactan chain could play a major role in the binding.

Table 15. Viscometric study at different time intervals to test if the endo-1,4-β-glucanase used was contaminated with an enzyme capable of causing breakdown of pectin such as pectin lyase (PL) or polygalacturonase (PG) enzymes. Control treatments have the appropriate buffer without the enzyme. 0.5% Pectin or xyloglucan were used. Treatments of (a) xyloglucan or (b) pectin with endoglucanase were tried over different time intervals. Treatment of (c) pectin with Pl. and PG was also tested as a control for the change in viscometric behaviour of pectin when degraded. This was further tested in Table 16.

Treatment	Time in viscometer (sec)
(a)	
Control xyloglucan (no enzyme)	764.39
Xyloglucan + endoglucanase (0min)	763.49
Xyloglucan + endoglucanase (30min)	56.00
Xyloglucan + endoglucanase (60min)	56.00
(b)	
Control Pectin (no enzyme)	140.00
Pectin + endoglucanase (0 min)	139.00
Pectin + endoglucanase (15 min)	132.00
Pectin + endoglucanase (30 min)	131.79
Pectin + endoglucanase (90 min)	129.55
Pectin + endoglucanase (18 hrs)	129.52
(c)	
Pectin + PG + PL (0 min)	129.52
Pectin + PG + PL (30 min)	49.58

Table 16. Treatment of pectin with (a) Pl. or (b) PG was further tested as a supplementary control for Table 15 to test the change in viscometric behaviour of pectin when degraded. Viscometric study at different time intervals was performed. 1% Pectin was used instead of 0.5% in order to observe the change in viscometry clearer.

Treatment	Time in viscometer (sec)
(a)	
Pectin + polygalacturonase (0 min)	296.20
Pectin + polygalacturonase (20 min)	88.34
Pectin + polygalacturonase (30 min)	83.68
(b)	
Pectin + pectin lyase (0 min)	282.90
Pectin + pectin lyase (20 min)	52.18
Pectin + pectin lyase (30 min)	51.17

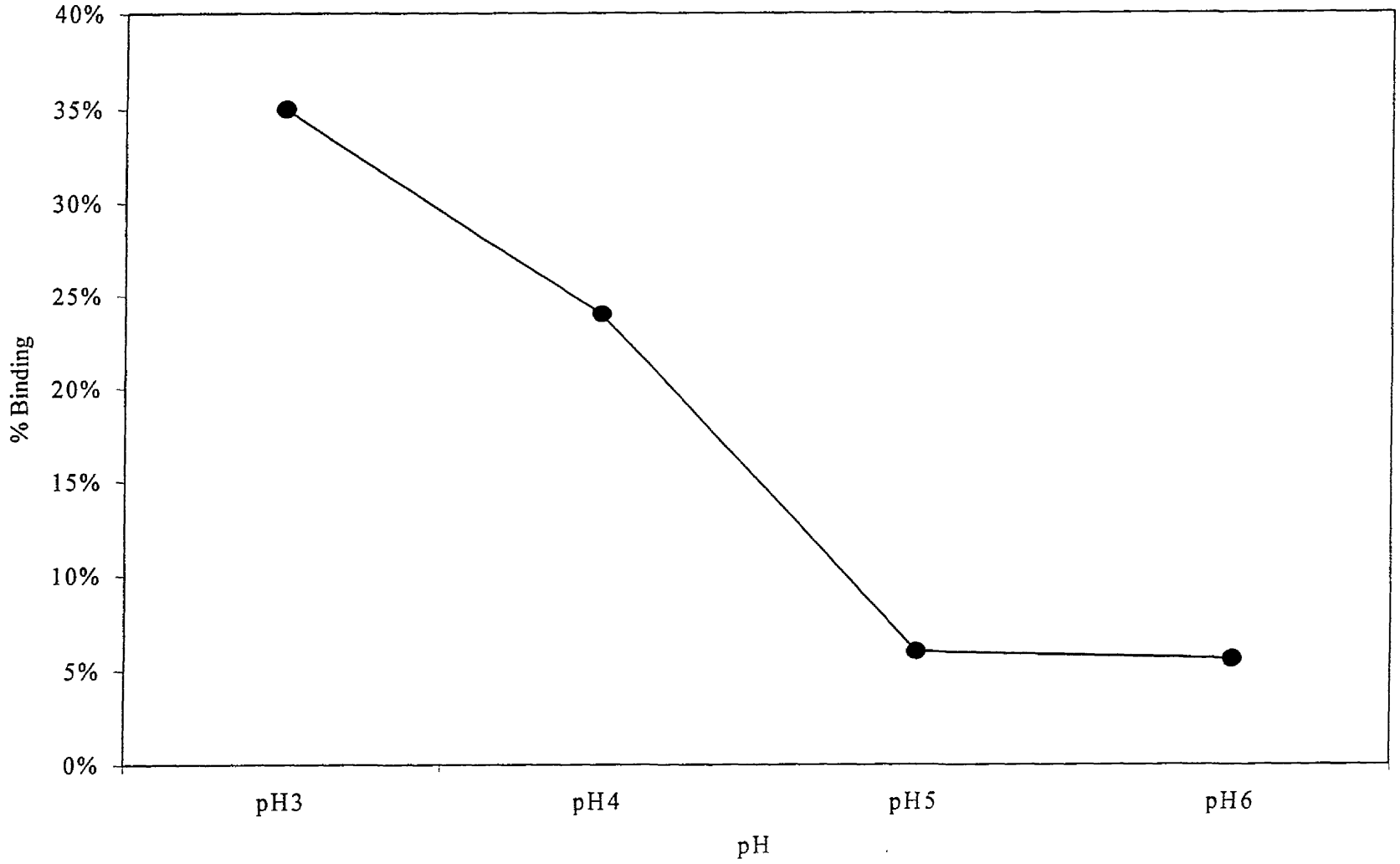

Fig 62. Binding of the high molecular weight EDTA extract from the particulate enzyme preparation to the cell wall (see Materials and Methods) at different pH.

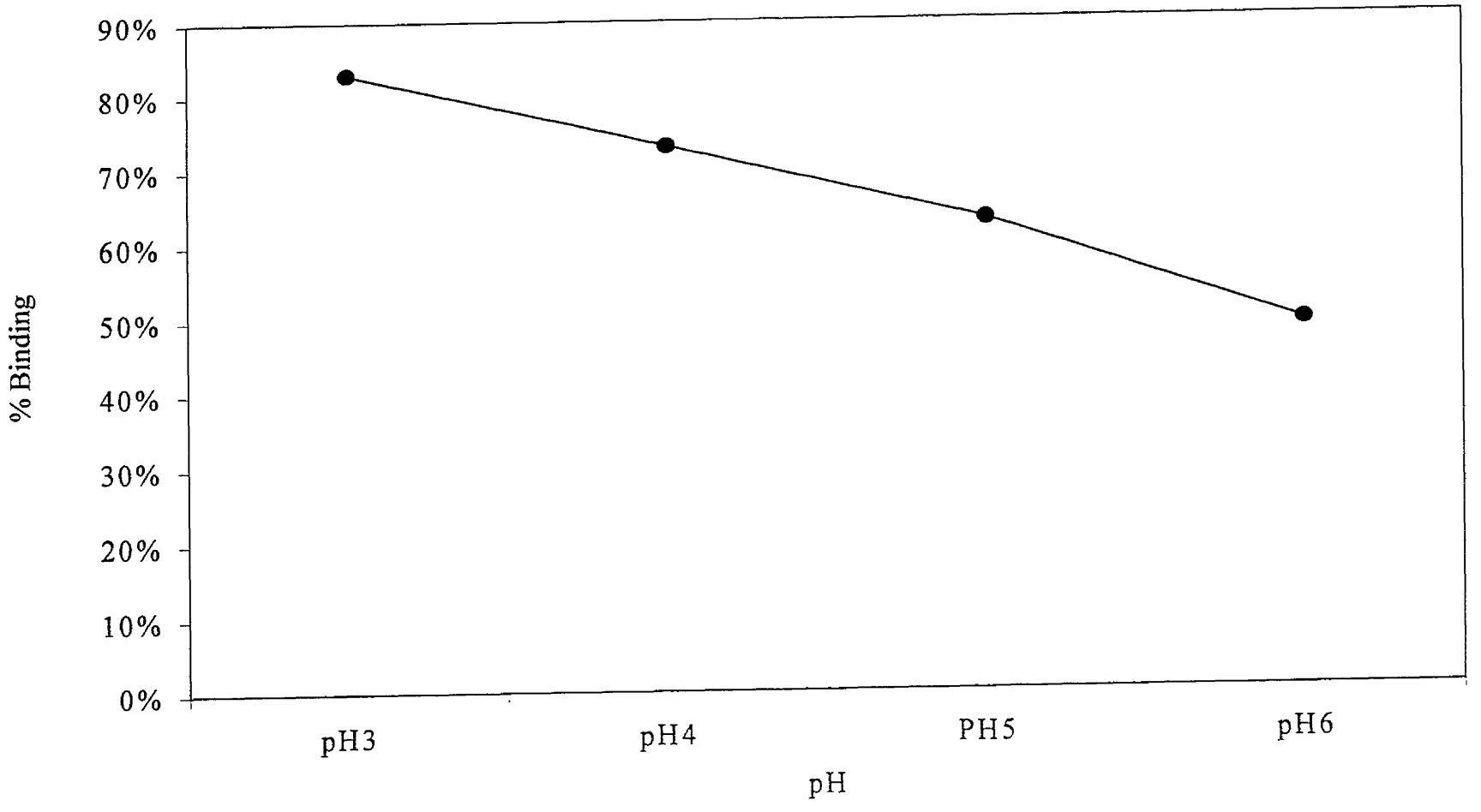

Fig 63. Binding of the high molecular weight EDTA extract from the particulate enzyme preparation to xyloglucan prepared from peas (see Materials and Methods) at different pH.

Table 17. Effect of different enzyme treatments on binding of the high molecular weight EDTA extract prepared from the particulate enzyme preparation to xyloglucan preparations at pH 3. Control treatment includes buffer with no enzyme.

Enzyme Treatments of High Molecular Weight EDTA extract	% Binding to xyloglucan at pH 3
Control	44%
Galactanase	1%
Polygalacturonase	1%
Endo-1,4-β-glucanase	5%
Pectin Lyase	10%
Bacterial Protease	44%

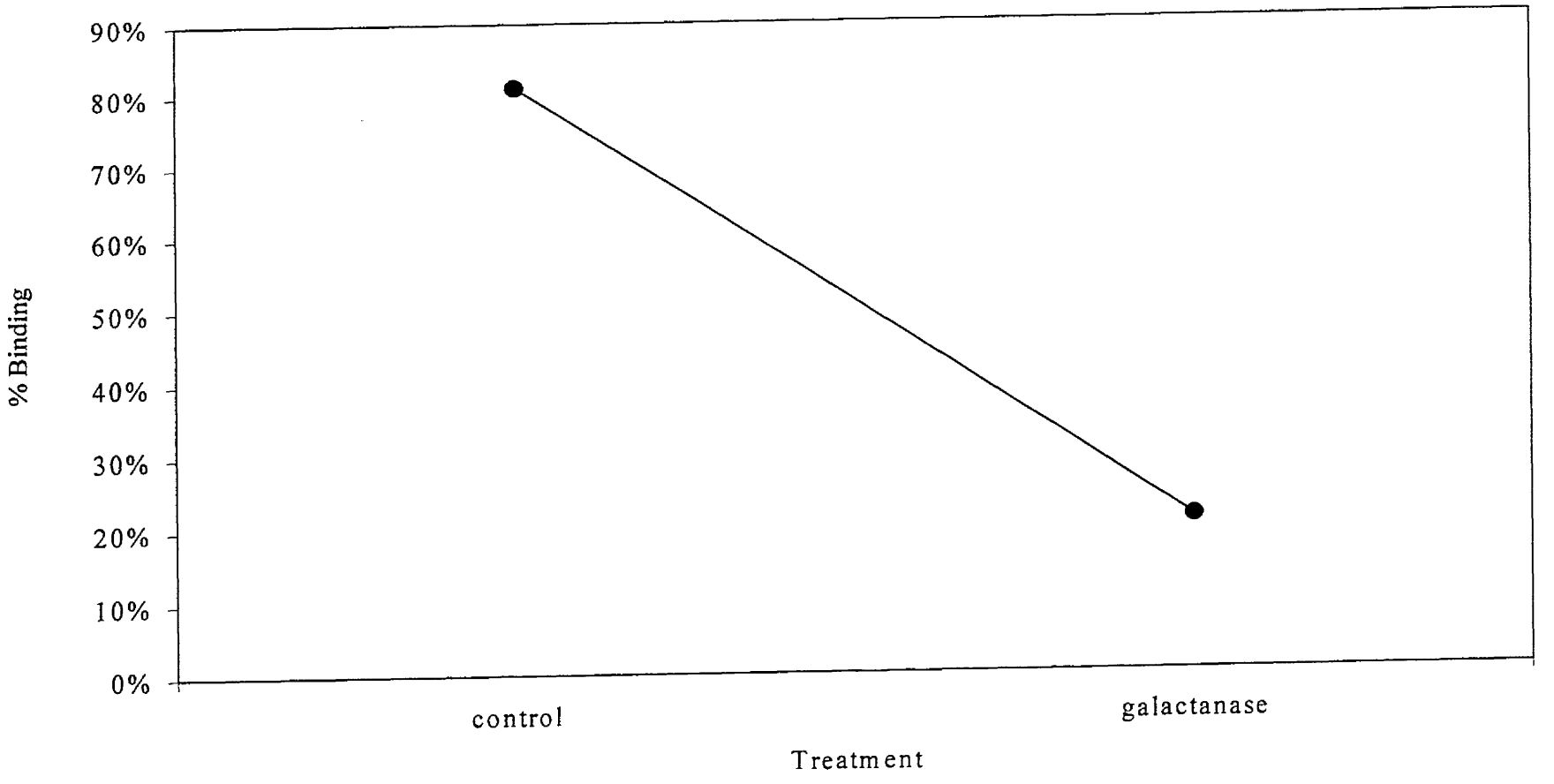

Fig 64. Binding of the treated-radioactive pectin with xyloglucan at pH 3. Radioactive pectin was prepared by incubating the particulate membrane preparation with UDP-[^{14}C]-glucuronic acid and collecting the high molecular weight EDTA extract from the particulate product. The radioactive pectin was treated with galactanase enzyme or the corresponding buffer (control) before binding it to xyloglucan. Xyloglucan was prepared from peas (see Materials and Methods). Control treatments were incubated with sodium acetate buffer (pH 4.5) at 40°C for 18 hrs as the enzyme-treated samples but without the enzyme.

The high molecular weight EDTA extract ([^{14}C]-galactose labelled) was run on an anion-exchange column. The eluted neutral and acidic fractions were dialysed and concentrated before binding to xyloglucan at pH 3 and pH 7 [Table 18]. Both fractions were bound in a pH dependent manner.

Conclusion

This chapter shows that the enzyme 1,4-β-galactan synthase, optimised in this investigation, transferred galactose residues to a large macromolecular complex containing galactan, RGI, PGA and xyloglucan. The bonds between these polymers were most probably covalent since they remain attached even after elution with 8mol.l^{-1} urea. The binding of this complex to the cell wall or in particular to xyloglucan appeared to require the galactan chain, and it acted in a pH-dependent manner.

Table 18. Binding of neutral and acidic fractions of the high molecular weight EDTA extracts run on ion-exchange to xyloglucan preparations at pH 3 and pH 7.

High Molecular Weight EDTA extract Fractions at different pH	% Binding to xyloglucan
Neutral fraction, pH 3	45%
Acidic fraction, pH 3	51%
Neutral fraction, pH 7	6%
Acidic fraction, pH 7	18%

Chapter VI

Discussion

Chapter VI

Discussion:

Our results demonstrate that pea microsomal membranes contain galactan synthase activity that is capable of transferring label from UDP-[^{14}C]-galactose to 1,4-β-galactan chains. The pH and divalent cation requirements for optimal activity were similar to those reported for mung beans (*Vigna radiata*; Allison and Reid, 1998) and potato suspension cultured cells (Geshi *et al.*, 2000). In contrast, Goubet and Morvan (1993, 1994) gave evidence for the presence of two galactosyltransferases in flax cells; at pH 5.0 the product was found to be a β(1→3), β(1→6) galactan, while at pH 8.0 the product was a 1,4-β-galactan. The optimal synthesis for the galactan product in this study was in the presence of Mn^{+2}. The requirement for divalent cations has also been observed with other polysaccharide-synthesising enzyme activities (Doong *et al.*, 1995; Geshi *et al.*, 2000; Baydoun *et al.* , 2001).

Previous work showed that galactose may be incorporated into several products, including xyloglucan and glycoprotein as well as galactans. In this work, conditions were chosen that gave maximum incorporation of radioactive galactose into products digestible with 1,4-β-galactanase. The presence of an endogenous galactanase enzyme caused the degradation of the products over time, in controls to which no exogenous enzyme had been added. This effect was decreased by reducing the incubation time of the enzymes. This endogenous galactanase enzyme may be located within the membrane preparations where synthesis and degradation of galactan may be occurring. Partial acid hydrolysis and analysis of the products of 1,4-β-galactanase activity

confirmed the formation of 1,4-β-galactan. The particulate enzyme preparation was extracted with EDTA-phosphate buffer (50mmol.l^{-1}, pH6.8) resulting in the solubilisation of 30%-50% of the radioactivity in the particulate product. Running the extracts on a Bio-Gel P10 column revealed that the extracts contained radioactivity in both polymeric material, excluded from the column, and low-molecular-weight material. Galactanase treatment prior to gel filtration decreased the radioactivity in the high-molecular-weight material by up to 70%-95% in different experiments [Fig 41 and Fig 49], indicating that most of the radioactivity in the high-molecular-weight material was in the form of a 1,4-β-galactan chain.

Rhamnogalacturonase B [Fig 51] degraded the high molecular-weight-product to products of Kav 0.78, rather larger than the products of 1,4-β-galactanase digestion (Kav 0.92). This indicates that the galactan chains were attached to RGI, as previously reported by Geshi *et al.* (2000) for potato suspension cultured cells. Degradation of the same high-molecular-weight material by pectin lyase and polygalacturonase to larger products with a broad range of sizes indicated that this polymeric material also included polygalacturonan. The acidic nature of the majority of the polymer was confirmed by anion-exchange chromatography. The binding characteristics were the same as those reported for pea cotyledon and carrot root pectin by Willat *et al.* (1999) and McCartney *et al.* (2000). Hence it can be concluded that the majority of the 1,4-β-galactan chains formed in our system were synthesised attached to the complex PGA-RGI polymer known to exist in most primary cell walls. The smaller amount of 1,4-β-galactan product that does not bind to the anion-exchange column may result from cleavage from the initial complex product, since the membrane preparation was shown to contain some endogenous 1,4-β-galactanase activity.

Partial breakdown of the polymeric product by endo-1,4-β-glucanase was also observed. One explanation for this could have been that the membrane preparations may have contained a UDP-galactose : xyloglucan galactosyltransferase, forming the galactose-(1→2)β-xylose bond characteristic of xyloglucans. This enzyme has been identified in peas (Faïk *et al.*, 1997). However, a number of lines of evidence point to an alternative explanation, that the radioactive polymer formed contained 1,4-β-galactan attached to a complex polymer containing xyloglucan as well as the PGA-RGI backbone of pectin:

1. Since at least 70% of the radioactivity in the polymeric product was in the form of 1,4-β-galactan, and more than half of it was degraded by endo-1,4-β-glucanase, some of the 1,4-β-galactan must have been part of a polymer containing also xyloglucan.

2. In the same way, since up to 90% of the radioactivity in the polymer bound to the anion-exchange column, some of the radioactivity degraded by endo-1,4-β-glucanase must have been part of a negatively-charged molecule.

3. The radioactive fragments formed by endo-1,4-β-glucanase digestion were considerably larger than the hepta- and nona-saccharides released from xyloglucan by this enzyme. The size of the fragments was not decreased by prolonged digestion with endo-1,4-β-glucanase for up to 18 hours.

4. The effects of polygalacturonase and endo-1,4-β-glucanase were not additive.

5. The material that bound to the ion-exchange column could also be labelled from UDP-[U-^{14}C]xylose or GDP-[U-^{14}C]fucose. Both xylose and fucose are

component sugars of xyloglucan, and some of these charged products were susceptible to endoglucanase digestion. Hence it is likely that these precursors were incorporating radioactive sugars into xyloglucan attached to an acidic component such as pectin.

The remaining 5-20% of the polymeric product was apparently not digested by any of the enzymes used. It may have been glycoprotein (Baydoun *et al.*, 2001). Incorporation of radioactivity into the galactose-xylose linkage of xyloglucans may not have occurred under the conditions used in this work, or alternatively any such radioactive xyloglucan products may have been insoluble in EDTA-phosphate buffer. The bonds linking xyloglucan to pectin in the complex product are not known. However, Thompson and Fry (2000) have presented strong evidence that xyloglucan does not form close non-covalent associations with pectin, and hence that the pectin-xyloglucan complexes found in rose cell walls were covalently linked. Rizk *et al.* (2000) have shown that nascent pectin binds non-covalently to xyloglucan by a mechanism which depends on the presence of an "assemblin" protein. However, this binding only occurs at pH 3-4, and no change in the elution behaviour of the radioactive polymeric product was observed when the polymer was passed through Bio-Gel P-10 at pH 6 or in the presence of 8 $mol.l^{-1}$ urea. Hence the links between xyloglucan and pectin in the radioactive product were probably covalent.

Thus it seems likely that the 1,4-β-galactan synthase, known to be located in the Golgi apparatus (Baydoun *et al.*, 2001), transfers galactose residues to a large macromolecular complex containing galactan, RGI, PGA and xyloglucan. Possible models for the

attachment of this complex are shown in Fig 65. This implies that such large complexes, recently shown to be present in the cell wall (Thompson and Fry, 2000; Femenia *et al.*, 1999), are formed in the Golgi and exported as such to the wall, rather than being built up from smaller precursors by enzymes such as transglycosylases in the cell wall. If this is a general feature of primary cell wall synthesis, it would imply that the matrix of the wall may be built up by association of large, covalently-linked "cassettes" containing pectin and xyloglucan and perhaps also glucuronoarabinoxylan (Femenia *et al.*, 1999) and protein (Rizk *et al.*, 2000), i.e. all the main matrix components of the Type 1 primary cell wall. The structural "independence" of the pectin and cellulose/hemicellulose networks would then arise secondarily, by selective covalent bond cleavage. It should be noted that the hot EDTA-phosphate extract used in this study may extract more material than ionically bound pectin, since the extraction was carried out at 100°C. Thus degradation of some bonds may have occurred. Other techniques such as extraction with imidizole or CDTA (Carrington *et al.*, 1993; Rihouey *et al.*, 1995) could have been used. The 4% KOH/0.1$NaBH^4$ extraction released a polymer that is more tightly bound to the membrane preparations. This extracted fraction is not hemicellulose since we are not dealing with the whole cell wall, hence it may be a complex similar to the one in the EDTA extract, but held more firmly to the membranes by aggregation to a protein.

The galactan complex was found to bind to the cell-wall and to its major hemicellulose, xyloglucan, in a pH-dependent manner with the highest binding at pH 3, which corresponds to the pH of a growing cell wall (McQueen-Mason, 1995). This pH-dependent binding was reported previously between glucuronoarabinoxylan GAX (Brett *et al.*, 1997) or pectin (Rizk *et al.*, 2000) and hemicelluloses from pea epicotyls.

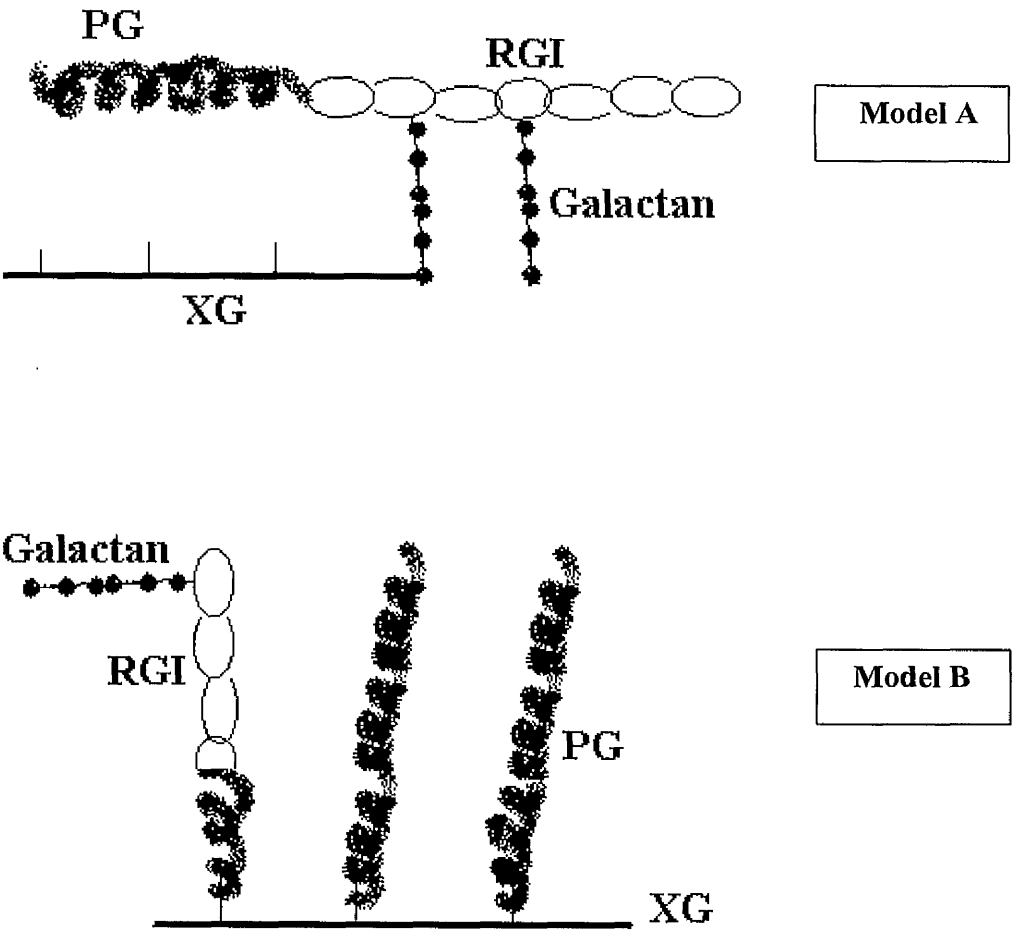

Fig 65- Models for possible attachment of xyloglucan (XG), polygalacturonan (PG), rhamnogalacturonan I (RGI),and galactan in our system. Different acidic side-chains may replace the some PG side-chains in model B.

Pre-treatment of the galactan complex with endogalactanase or polygalacturonase showed a decrease in binding. Whereas, pre-treatment with bacterial protease did not reveal a decrease in binding contrary to the results obtained by Rizk *et al.*, 2000. Thus the cell wall appears to adopt different mechanisms in binding pectin non-covalently to the cell wall: either with the help of an "assemblin" protein or through a galactan chain covalently attached to a pectic backbone. The latter possibility may be of major interest especially in the study of elongation or ripening where solubilisation of galactan is quite characteristic (Seymour *et al.*, 1990). During low pH, as that during elongation, the pectic galactan chain may bind tightly to xyloglucan thus competing with the cellulose-xyloglucan binding making the cell wall more flexible. More work should be done to study the involvement of this binding to ripening.

Chapter VII

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Appendix

Chapter III: conditions after 13,000 *g* initial centrifugation.

a) Optimisation conditions (Objective 1)

- Cation effect:	Mn^{+2}, 10mmol.$^{-1}$
-UDP-xylose, UDP-glucose:	inhibitory
-Time-course:	60 min
-pH Effect:	broad peak
-UDP-[^{14}-C]-Galactose:	1.5KBq
-Termination method:	Needs ethanol and standard washes

b) Analysis of the product

-TFA analysis:	Galactose product (objective 2)
-Enzyme treatments:	Complex structure

Chapter IV: conditions after 27,000 *g* initial centrifugation.

a) Optimisation conditions (objective 1)

-Centrifugation speed:	27,000 *g* optimal
-Non-radioactive UDP-Gal:	0.2mmol.$^{-1}$
-pH effect:	pH 6,7 for particulate product; pH 5.5 for galactan product

-No additional washes needed after standard washes
-No exogenous acceptors increased incorporation into high molecular weight EDTA

b) Analysis of the product

-Partial TFA:	Galactan oligomers (objective 2)
-EDTA/KOH extract:	EDTA solubilises more high molecular weight material

-Galactanase treatment: (objective 2)

* Presence of endogenous galactanase (caused breakdown of control)

*Low molecular weight after galactanase treatment of high molecular weight EDTA has galactobiose

Chapter V:

Identification of Galactan chain:

-Evidence for oligosaccharide series of galactan:(objective 2)

* Time-course incubation
* EDTA extract

-Protease treatment:	gave no intermediate peaks on CL-6B
-Different enzyme treatments:	gave complex made of pectin (HG/RGI)-galactan-XG [Fig 49,50,51] (objective 3)
-Anion-exchange:	attachment to acidic polymer, strong attachment withstands Urea (objective 3)
-Binding to the cell wall:	pH-dependent binding (objective 4)
-Viscometric studies:	endo-glucanase not contaminated with pectin-degrading enzyme