Journal of Chromatography, 238 (1982) 183–192
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,591

MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY OF LEOZYM, A PECTIC ENZYME PREPARATION, ON ION-EXCHANGE DERIVATIVES OF SPHERON

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(Received November 30th, 1981)

SUMMARY

Leozym, a commercial pectic enzyme preparation, was subjected to medium-pressure liquid chromatography on weakly, medium, and strongly acidic cation exchangers and on weakly and strongly basic anion exchangers, and to hydrophobic chromatography on the ionogenically unsubstituted glycomethacrylate macroreticular gel of the Spheron type. The course of the gradient chromatography was examined by continuous measurement of the absorbance of the effluent at 285 and 254 nm, by pH and conductivity measurements on the fractions collected and by specific measurements of selected enzyme activities. Fractions containing endo-D-galacturonanase, pectin-lyase and pectin-esterase activity were successfully resolved. The presence of multiple forms of endo-D-galacturonanase and pectin-lyase in the preparation examined was shown. The technological importance of these findings is briefly discussed.

INTRODUCTION

Commercial pectic enzymes used in the canning industry contain components showing the activity of endo-D-galacturonanase*, exo-D-galacturonanase, pectin-lyase and pectin-esterase. During the past few years, in order to increase the efficiency of production and quality, there has been a trend towards the use in certain industrial processes of exclusively enzyme preparations that contain certain components of the pectic complex only; such preparations are composed of, *e.g.*, pectin depolymerases

* Nomenclature of enzymes and abbreviations used: endo-D-galacturonanase and exo-D-galacturonanase, poly(1–4)-α-D-galactosiduronate glycanohydrolase, E.C. 3.2.1.15 and E.C. 3.2.1.67; pectin-lyase, poly(methyl-D-galactoside uronate)lyase, E.C. 4.2.2.10; pectin-esterase, pectin pectyl-hydrolase, E.C. 3.1.1.11; the earlier trivial names of the first two enzymes were endopolygalacturonase and exopolygalacturonase; HPLC, high-pressure liquid chromatography; MPLC, medium-pressure liquid chromatography.

0021-9673/82/0000–0000/$02.75

only, or show an increased content of a certain pectic enzyme, such as endo-D-galacturonanase or pectin-lyase. The preparation and analysis of these commercial products require rapid and efficient procedures that permit one or several enzymes to be separated from the original mixture. Of the laboratory procedures used so far for the separation or purification of microbial pectic enzymes, only bioaffinity chromatography, which permits the selective resolution of endo-D-galacturonanase[1] and pectin esterase[2], appears to be possibly useful.

During the past few years high- and medium-pressure liquid chromatography have been used for the separation of biopolymers[3–5]. For such separations of enzymes[6] and certain other proteins[7] macroreticular ion exchangers with a matrix of a hydrophilic glycolmethacrylate gel (Spheron) have been employed. The possibilities for their application in the analytical separation of pectic enzymes from two commercial preparations. Pectinex Ultra (AG Ferment, Basle, Switzerland) and Rohament P (Röhm and Haas, Darmstadt, G.F.R.), by medium-pressure liquid chromatography have been discussed earlier[8].

This study was designed to examine the applicability of the procedures developed for the separation of pectic enzymes present in Leozym (Slovlik, Leopoldov, Czechoslovakia); this preparation is obtained in a pilot plant from fermented media of *Aspergillus niger*, which is a waste product from citric acid manufacture. Leozym contains endo-D-galacturonanase as the main component, together with pectin-lyase, pectin-esterase and trace amounts of exo-D-galacturonanase. The preparation is used predominantly for the processing of fruits and vegetables. The analytical procedures developed should provide information on the prospective large-scale separation on Spheron derivatives.

EXPERIMENTAL

Materials

Enzyme. Leozym contained 19.7% of protein, pectic enzymes endo-D-galacturonanase (0.188 katal/kg), exo-D-galacturonanase ($5 \cdot 10^{-7}$ katal/kg), pectin-esterase ($1.5 \cdot 10^{-7}$ katal/kg)[9] and pectin-lyase, the activity of which cannot be determined in the crude preparation because of the large amounts of coloured contaminants (the activity of the enzymes is expresed in moles of reducing groups or carboxyl groups liberated in 1 sec).

Ion exchangers. Spheron C-1000 (1.85 mequiv./g), Spheron Phosphate-1000 (3.1 mequiv./g), Spheron S-1000 (1.72 mequiv./g), Spheron DEAE-1000 (1.5 mequiv./g), Spheron TEAE-1000 (1.4 mequiv./g) and unsubstituted Spheron 1000 (0.044 mequiv./g) were products of Lachema (Brno, Czechoslovakia). The particle size of all products was 25–40 μm, except for the phospho derivative (40–60 μm).

Substrates. Citrus pectin, employed for the determination of the activity of pectin esterase, degree of esterification (DE) 65.1%, was a preparation obtained from commercial Genu Pectin. Type B (Københavns Pektinfabrik, Copenhagen, Denmark) by washing with 60% ethanol containing 5% hydrochloric acid and then with 60 and 96% ethanol. Pectic acid used for the determination of the activity of endo-D-galacturonanase was prepared from purified citrus pectin by repeated alkaline deesterification in 0.1 *M* sodium hydroxide solution, followed by precipitation at pH 2.5. Highly esterified pectin (DE 93.8%) was prepared by esterification of pectic acid in a 1 *M* solution of sulphuric acid in methanol[10].

Methods

Chromatographic methods. The general principles of the work with Spheron ion exchangers and the medium-pressure liquid chromatography apparatus have been described previously[3,6,11]. The technique of rapid chromatographic analysis of pectic enzymes has been described elsewhere[8].

Assays. The activity of endo-D-galacturonanase was examined in terms of increase in reducing groups by the method of Somogyi[12] after a 10-min incubation of a reaction mixture composed of 0.8 ml of a 0.5 % solution of pectic acid in 0.1 *M* acetate buffer (pH 4.2) and 0.2 ml of the effluent fraction. The activity of pectin-esterase was determined by titration with 0.1 *M* sodium hydroxide solution of carboxyl groups liberated during a 60-min incubation of a mixture of 5 ml of a 0.5 % solution of pectin in 0.1 *M* acetate buffer (pH 4.4) and 1 ml of the effluent from the column. The activity of pectin-lyase was determined in terms of increase in absorbance at 235 nm[13] of a reaction mixture containing 2.5 ml of a 0.5 % solution of highly esterified pectin in 0.1 *M* acetate buffer (pH 5.6) and 0.5 ml of the effluent fraction.

RESULTS

The possibilities of using MPLC on Spheron and its ion-exchange derivatives for the separation of pectic enzymes in Leozym were examined under experimental conditions similar to those described in the preceding study[8] dealing with the separation of enzymes from Pectinex Ultra and Rohament P. Because of the enzyme composition of the preparation examined and the character of the microbial producer (*Aspergillus niger*), we expected that the behaviour of the enzymes would be similar to that of enzymes in Pectinex Ultra. The preparation was chromatographed on Spheron 1000 and on its ion-exchange derivatives: weakly, medium and strongly acidic cation exchangers and on weakly and strongly basic anion exchangers.

Pectin-esterase S was well separated on the weakly acidic carboxyl cation exchanger Spheron C as the middle peak (Fig. 1) from a large amount of endo-D-galacturonanase N. The tailing, small peak of pectin-lyase L indicates considerable sorption of this enzyme by the resin. Enzymes S and N were eluted from the column in the same order and by the same buffer concentrations as with Pectinex Ultra (Fig. 2 in ref. 8). Endo-D-galacturonanase N, however, formed in Leozyme only one fraction, which was identical with the major fraction of Pectinex Ultra. The position of the peak of pectin-lyase, however, was entirely different. It emerged as the first peak when Leozym was chromatographed whereas with Pectinex Ultra it was contained in the last peak.

Chromatography on the medium acidic cation exchanger Spheron-phosphate (Fig. 2) resulted in the separation of pectin-esterase S, present in the most retarded peak, from the mixed peak of pectin-lyase L and the main fraction of endo-D-galacturonanase N. These two enzymes were not separated, however, even when Pectinex Ultra was chromatographed (Fig. 3 in ref. 8; see also the remark on p. 102 of ref. 8).

A partial separation of all three pectic activities was achieved on the strongly acidic sulpho acid cation exchanger Spheron-S (Fig. 3). The course of the separation and the order of the enzymes eluted, *i.e.*, pectin-esterase S, pectin-lyase L, endo-D-galacturonanase N, shows that this ion exchanger in combination with a milder elution gradient could be used for the isolation of specific preparations from Leozym.

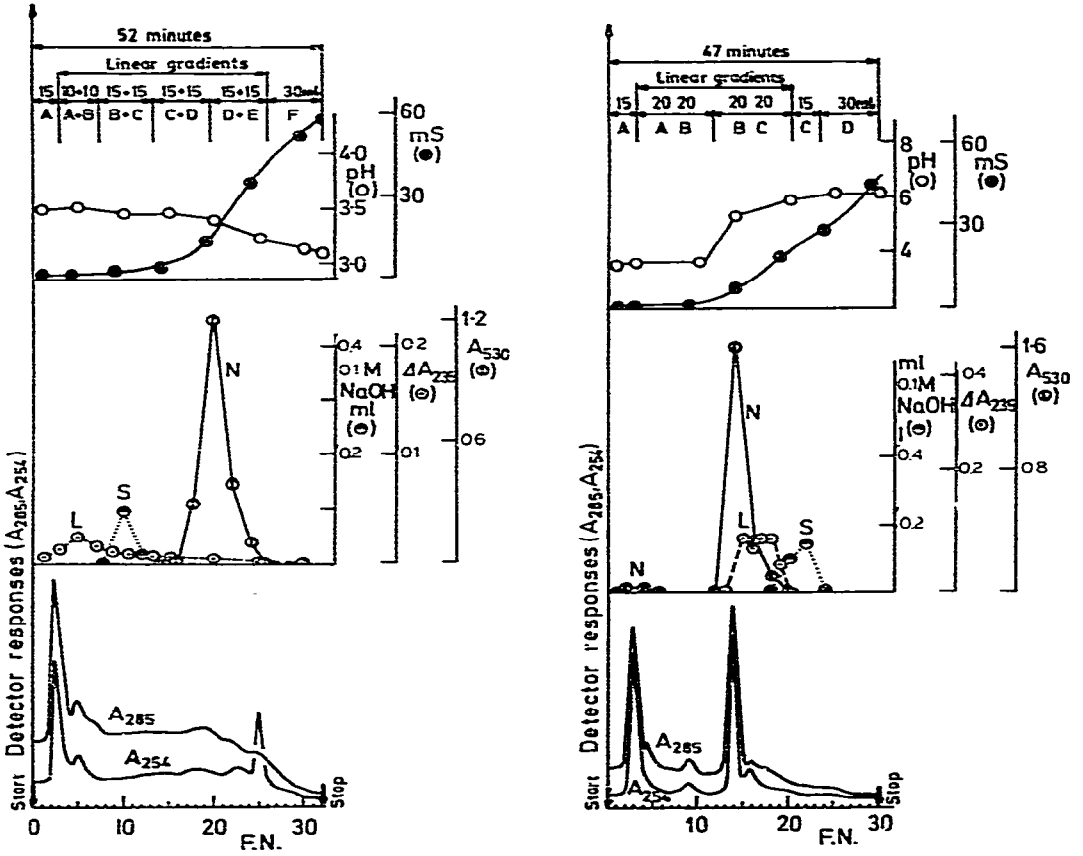

Fig. 1. Chromatography of Leozym (25 mg) on Spheron C-1000 (20 × 0.8 cm; particle size 25–40 μm). Enzyme activities: L, pectin-lyase; N, endo-D-galacturonanase; S, pectin-esterase. The electrical conductivity is given in millisiemens. The buffers were prepared from sodium hydroxide of the given final concentration whose pH was adjusted to 3.5 with formic acid: A, 0.05 *M*; B, 0.1 *M*; C, 0.2 *M*; D, buffer C, 1 *M* in sodium chloride; E, unbuffered 2 *M* sodium chloride solution was used for preliminary regeneration of the column and for washing out any protein remaining in the column. Flow-rate, 3 ml/min; temperature, 25°C. counterpressure, 4 atm. Fractions (4.5 ml) collected at 90-sec intervals. F.N. = Fraction number.

Fig. 2. Chromatography of Leozym (25 mg) on Spheron-phosphate 1000 (40–60 μm). Counterpressure, 6 atm; other details as in Fig. 1. Buffers: A, 0.005 *M* ammonia solution + formic acid (pH 3.5); B, 0.3 *M* ammonia solution + acetic acid (pH 6); C, 1 *M* ammonia solution + acetic acid (pH 8), 0.5 *M* in sodium chloride; D, 2 *M* sodium chloride solution.

A better separation was obtained with Pectinex Ultra (Fig. 4 in ref. 8). The pectin-lyase L and endo-galacturonanase N present in both Leozym and Pectinex Ultra were resolved into multiple forms, but only one member of the pair was identical in both preparations (peak L in fraction 12 and the highest peak N in both figures). In contrast, the second peak L emerged last (Fig. 3) rather than first as in the earlier study (Fig. 4 in ref. 8). The positions of the peaks of pectin-esterase S in the elution patterns obtained with the two preparations were different.

The above similarities in the behaviour of certain enzymes in Pectinex Ultra and Leozym, especially during their chromatography on the weakly acidic cation

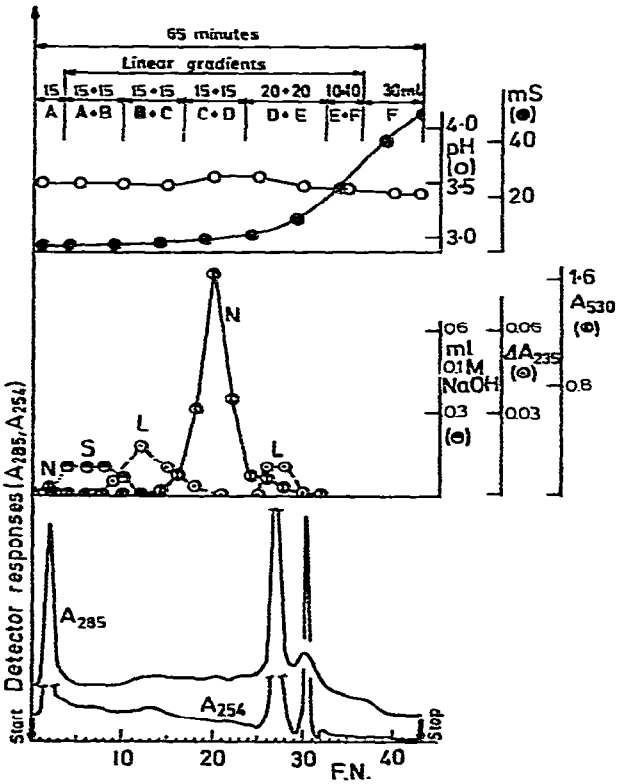

Fig. 3. Chromatography of Leozym (25 mg) on Spheron S-1000. Counterpressure, 8 atm; other details and buffers A, B and C as in Fig. 1. The remaining buffers were prepared from sodium hydroxide of the given final concentration, whose pH was adjusted to 3.5 with formic acid; D, 0.3 *M*; E, 0.5 *M*, 1 *M* in sodium chloride; F, unbuffered 2 *M* sodium chloride solution.

exchanger (Fig. 1), were not observed when these preparations were chromatographed on anion exchangers. The chromatography of Leozym on the weakly basic Spheron-DEAE (Fig. 4) resulted in a poorer separation of enzymes than that obtained with Pectinex Ultra (Fig. 7 in ref. 8) where all the enzymes present, including the two forms of endo-D-galacturonanase, were well separated in Tris–hydrochloric acid buffer at pH 7. The position of the peak of Leozym endo-D-galacturonanase N in Fig. 4 does not correspond to the position of any of the multiple forms N observed in Pectinex Ultra (Fig. 7 in ref. 8). A decrease in the pH of the elution buffer to pH 5, which is convenient with respect to the stability of microbial pectic enzymes, led to unfavourable retardation of pectin-esterase and thus to a shift to a peak containing the remaining two enzymes. In this experiment, not illustrated here, the minor form of endo-D-galacturonanase N emerged only at the beginning of the chromatogram; this form was obviously overlapped by the major form and emerged in one peak (N) under the conditions used to obtain Fig. 4.

A similar course of chromatography can be seen in Fig. 5, which illustrates experiments with the strongly basic (quaternized) anion exchanger Spheron-TEAE. The pattern of three separated enzymes, S, L and N, resembles that obtained with the

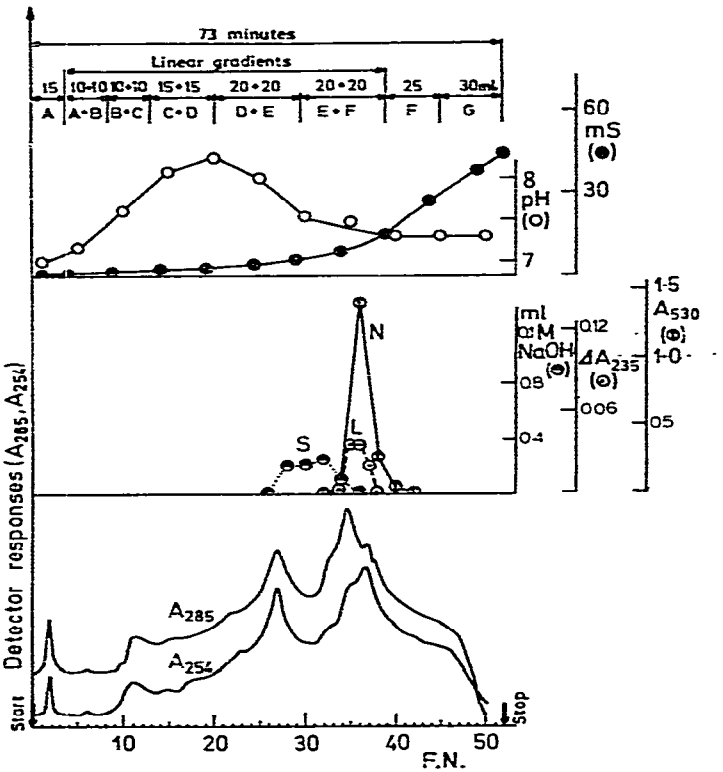

Fig. 4. Chromatography of Leozym (25 mg) on Spheron DEAE-1000. Counterpressure, 6–8 atm; other details as in Fig. 1. The buffers were prepared from hydrochloric acid of the given final concentration whose pH was adjusted to 7 with Tris: A, 0.005 *M*; B, 0.05 *M*; C, 0.1 *M*; D, 0.2 *M*; E, 0.4 *M*; F, buffer E, 1 *M* in sodium chloride; G, unbuffered 2 *M* sodium chloride solution.

weakly basic anion exchanger (Fig. 4). Another multiple form of endo-D-galacturonanase N was well separated. We cannot explain why this form was not separated also on the weakly basic anion exchanger (a separation was observed when Pectinex Ultra was chromatographed on Spheron-DEAE; *cf.*, Fig. 7 in ref. 8).

We considered it of interest to examine the behaviour of pectic enzymes on unmodified Spheron 1000, *i.e.*, on the matrix itself, as a comparison of this behaviour with the experiments with ion exchangers should provide information on the effects of the individual ionogenic groups. In earlier experiments with Pectinex Ultra and Rohament P (Figs. 1 and 9 in ref. 8) conditions similar to those employed for ion-exchange chromatography were used, the elution being started with solutions of low ionic strength. Under these conditions, however, it is difficult to distinguish the participation of ion exchange due to trace amounts of carboxyls (originating in the monomer used as the starting material in the production of Spheron and responsible for a cation-exchange capacity of 0.04 mequiv./g of Spheron 1000) from the participation of hydrophobic interactions (with aliphatic hydrocarbon chains of the matrix; *cf.*, Fig. 1 in ref. 7). Therefore, Leozym was chromatographed under conditions of hydrophobic chromatography: a support equilibrated in a buffer of high ionic strength was used and the latter was decreased in a gradient manner. Under these

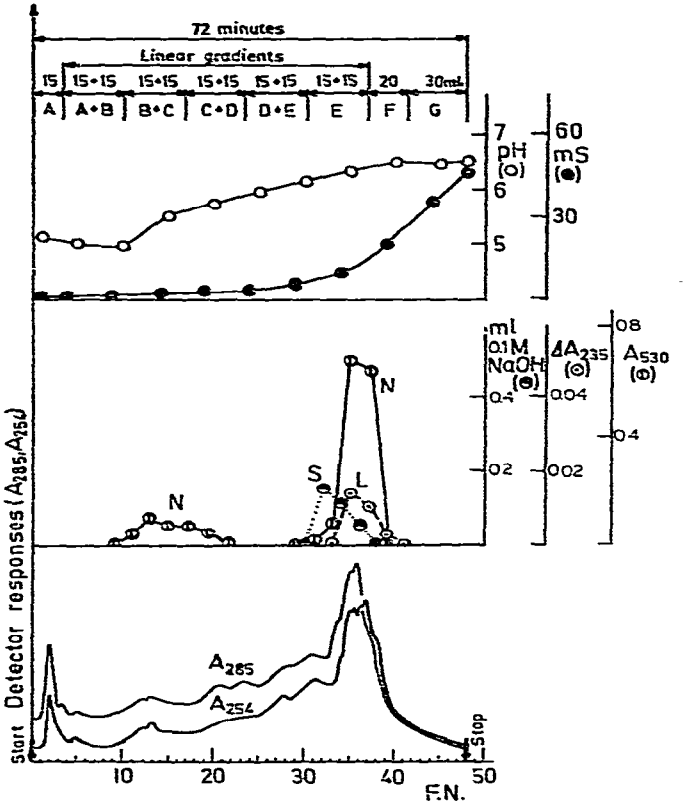

Fig. 5. Chromatography of Leozym (25 mg) on Spheron TEAE-1000. Counterpressure, 11 atm; other details as in Fig. 4, except that the pH was adjusted to 5.

conditions the proteins cannot be retained by the ionogenic groups, but are first "salted" on to the matrix and then the hydrophobic bonds are successively released during the chromatography.

The result is shown in Fig. 6. All of the enzymes present, *i.e.*, N, L and S, emerged almost in one peak in the void volume. Pectin-lyase L only was slightly retarded; this can be ascribed to its hydrophobicity, which is slightly higher than that of the remaining enzymes. The small peak of endo-D-galacturonanase N at the end of the chromatogram, emerging at a very low ionic strength, can be explained either by the existence of a multiple form, characterized by a strong hydrophobic bond to the matrix, or by the low capacity of hydrophobic bonds of the matrix capable of binding part of the hydrophobic form N only and leaving most of the enzyme to emerge in the void volume.

DISCUSSION

The results obtained in this study confirmed the advantages MPLC on ion-exchange derivatives of Spheron for the analysis of commercial pectic enzyme preparations mentioned previously[8]. These advantages are as follows:

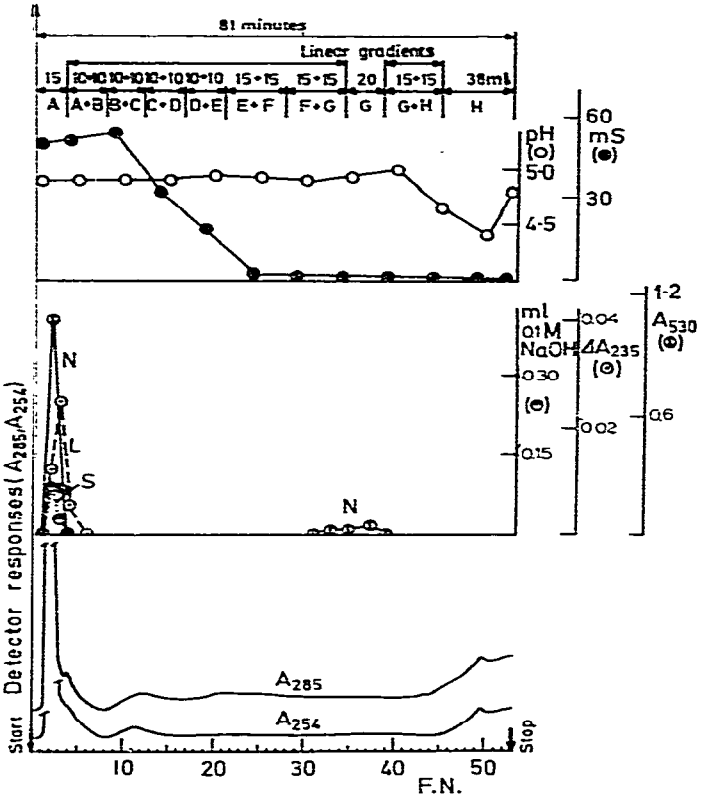

Fig. 6. Hydrophobic chromatography of Leozym on ionogenically unsubstituted Spheron 1000. Counter-pressure, 5 atm; other details as Fig. 1. The buffers were prepared from sodium hydroxide of the given final concentration whose pH was adjusted to 5 with acetic acid; A, 0.2 *M*, 2 *M* in sodium chloride; B, 0.1 *M*, 1 *M* in sodium chloride; C, 0.05 *M*, 0.5 *M* in sodium chloride; D, 0.05 *M*, 0.1 *M* in sodium chloride; E, 0.05 *M*; F, 0.01 *M*; G, distilled water; H, water–ethanol (3:1).

(a) Improved reliability of the determination of the individual enzyme activities as a result of the elimination of the simultaneous action of several enzymes on the same substrate, provided by the possibility of rapid separation of the enzymes. The simultaneous separation of the reducing compounds present in the preparation, which usually also emerge in the void volume, is possible. These compounds interfere in the analyses of the unfractioned preparation.

(b) The possibility of the determination of the presence and activity of pectinlyase by a spectrophotometric method and the higher sensitivity of the spectrophotometric determination of the activity of D-galacturonanases as a result of the separation of the coloured contaminants. This can be explicitly observed by a comparison of UV absorbance curves with the enzyme activity curves; this comparison shows that sometimes a completely negligible part of the absorbance of the commercial preparation can be accounted for by the enzyme present.

(c) The possibility of identifying multiple forms of pectic enzymes. According to these analyses commercial preparations of different origin can be distinguished chromatographically, thus establishing the technological regime of production, the

conditions of enzyme maturation and material handling and storage. Multiple forms need not necessarily be caused genetically or by differences in quaternary structure as is the case with numerous isoenzymes. They can result from complementary modification of originally homogeneous extracellular enzymes after secretion from the cell; such a modification can be due to limited proteolysis, changes in the sugar moiety of glycoproteins and conformational changes caused by temperature, ions or other compounds (the appearance of so-called conformers; see, *e.g.*, ref. 14). These modifications need not necessarily result in a decrease in the activity of the enzyme. Numerous enzymes are known with peptide chains interrupted at several sites; these enzymes retain full enzymatic activity. Such changes, however, are responsible for changes in electrophoretic or chromatographic behaviour and are readily detected by HPLC or MPLC. In general these methods are therefore of possible importance for the rapid checking of technological processes, enzyme maturation and storage and transportation of crude commercial enzyme preparations. Deviation from the permitted temperature and time limits usually manifests itself by the appearance of or increase in multiple enzyme forms with a simultaneous decrease in the enzyme activity[5]. The purification and characterization of two forms of vegetable pectin esterases has been described elsewhere[15].

The results obtained in this study show the potential use of MPLC in the preparation of products with modified contents of pectic enzymes and the possibilities of the preparation of specific products by MPLC. Likewise, preparations containing pectin-depolymerizing enzymes only, *i.e.*, free from pectin-esterase (which liberates methanol from pectin and can be the cause of toxicity of depectinized fruit juices, especially for children), could be obtained by removal of pectin-esterase via its sorption by a medium acid cation exchanger (Fig. 2), where it is retained. The use of the thus purified enzyme preparation in immobilized form would be economical for industrial application. Specific pectin esterase for research applications can be prepared by chromatography on a weakly basic anion exchanger (Fig. 4), from which it is eluted as the first peak, and in enriched form by chromatography on a weakly acidic cation exchanger (Fig. 1). The separation of all three enzyme activities could also be effected on a strongly acidic cation exchanger (*cf.*, Fig. 3) by rechromatography of the fractions.

The flow-rates used in this study (3 ml/min in columns of 0.8 cm diameter, *i.e.*, 5 ml/cm^2 · min) correspond to a total flow-rate of 30 l/min through a technical filter of 80 cm diameter at medium pressures of 5–10 atm. The time necessary for one run is around 1 h (and could be reduced for industrial applications). In general, this is very convenient in the production of enzyme preparations where the obtaining of high activities depends on the rate of processing. These are possibilities for future technical developments in the chromatography of enzymes. A necessary prerequisite of such developments, however, is the adequate production of chemically and biochemically stable, rigid, macroporous and hydrophobic ion exchangers at a reasonable cost.

ADDENDUM

While this manuscript was being prepared we obtained a reprint of the paper by Castino and Ubigli[16] describing the separation of two commercial pectic enzyme preparations and the characterization of the fractions obtained.

REFERENCES

1 Ľ. Rexová-Benková and V. Tibenský, *Biochim. Biophys. Acta*, 268 (1972) 187.
2 M. A. Vijayalakshmi, C. Bonaventura, D. Picque and E. Segard, in O. Hoffmann-Ostenhof (Editor), *Affinity Chromatography, Proc. Int. Symp.*, Pergamon Press, Oxford, 1978, p. 115.
3 O. Mikeš, *Int. J. Peptide Protein Res.*, 14 (1979) 393.
4 E. F. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
5 O. Mikeš, *Ernährung*, 5 (1981) 88.
6 O. Mikeš, P. Štrop and J. Sedláčková, *J. Chromatogr.*, 148 (1978) 237.
7 O. Mikeš, P. Štrop, J. Zbrožek and J. Čoupek, *J. Chromatogr.*, 119 (1976) 339.
8 O. Mikeš, J. Sedláčková, Ľ. Rexová-Benková and J. Omelková, *J. Chromatogr.*, 207 (1981) 99.
9 O. Markovič, Ľ. Rexová-Benková and K. Heinrichová, unpublished work.
10 W. Heri, H. Neukom and H. Deuel, *Helv. Chim. Acta*, 44 (1961) 1939.
11 O. Mikeš, P. Štrop, M. Smrž and J. Čoupek, *J. Chromatogr.*, 192 (1980) 159.
12 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19.
13 P. Albersheim, H. Neukom and H. Deuel, *Helv. Chim. Acta*, 42 (1960) 1422.
14 O. Mikeš, K. Worowski and J. Turková, *Collect. Czech. Chem. Commun.*, 38 (1973) 3339.
15 C. Versteeg, F. M. Rombouts and W. Pilnik, *Lebensm. Wiss. Technol.*, 11 (1978) 267.
16 M. Castino and M. Ubigli, *Frazionamento per Gel Filtrazione delle Attività Enzimatiche di Preparati Pectolitici Commerciali*, Istituto Sperimentale per l'Enologia di Asti, Tipolitografia Editrice Scarpis, Conegliano, Italy, No. 2, 1977, pp. 28.