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LIGAND-EXCHANGE CHROMATOGRAPHY OF NUCLEASES

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SUMMARY

The synthesis of chelating sorbents for ligand-exchange chromatography of enzymes is described. An inorganic support "Silochrom" and organic "Spheron", TSK-Gel HW 55 and cellulose were used as initial supports. The chelating sorbents contained iminodiacetic acid and iminodimethylphosphonic acid as stationary ligands. In order to obtain monofunctional sorbents, iminodiacetic acid was added in the form of its dimethyl ester. The concentration of stationary ligands on the sorbents varied from 10 to 100 μmol per ml sorbent. A chelating sorbent (in nickel form) was shown to be effective in the purification of exonuclease A5 from actinomyces. Electrophoretically homogeneous exonuclease A5 was obtained with a 25% yield. A chelating sorbent with iminodiacetic groups (in copper form) was applied to the isolation of endonuclease from *Serratia marcescens* directly from the culture medium. The capacity of the chelating sorbents for the endonuclease was studied as a function of the stationary ligand concentration. After one of stage of purification, more than 70% pure enzyme was obtained with a yield exceeding 80%.

INTRODUCTION

The technique of ligand-exchange chromatography was first used for the separation of proteins by Porath *et al.*[1] in 1975. In the last few years the use of this technique for the isolation and purification of proteins has greatly increased[2,3]. It is based on the different abilities of proteins to form complexes with transition metal ions, and involves functional groups, *e.g.*, imidazole or thiol, located on the surface of the proteins. The stationary phase is actually a chelating support which is electrically charged due to the presence of transition metal ions and interacts with the compounds to be separated by formation of coordination bonds. The major advantages of the technique are high selectivity for chelated proteins (determined by intrinsic properties of the proteins (determined by intrinsic properties of the proteins and the nature of the metal ions attached to the chalte-forming sorbent), stability of the complexes formed and the possibility to employ the same sorbent for isolation of different types of enzymes by choosing appropriate pH values and metal ions.

The present communication describes the synthesis of chelating sorbents based

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on "Silochrom" and the organic supports "Spheron", TSK-Gel HW 55 and cellulose, and their application to the isolation of highly purie exonuclease A5 from actinomyces and of endonuclease from the bacterium *Serratia marcescens*. In the latter case the cell culture medium was immediately loaded onto a chelating sorbent. Nevertheless, the sorbent retained its high selectivity for the enzyme.

MATERIALS AND METHODS

Silochrom S 80 (VNII Luminofor, Stavropol, U.S.S.R.) having a pore diameter of 68 nm, S_{sp} = 68 m^2/g and a particle size of 0.16–0.32 mm, TSK-Gel HW 55 (Toyo Soda, Japan) with a particle size of 30–60 μm, Spheron HEMA-1000 (Lachema, Czechoslovakia) and cellulose MN 300 (Whatman, U.K.) were employed. 3-(2',3'-Epoxypropoxy)propyltrimethoxysilane was obtained from Serva (F.R.G.), tris(hydroxymethyl)aminomethane from Reanal (Hungary), disodium *p*-nitrophenylphosphate from Feinchemie K.-H. Kallies KG (F.R.G.), total yeast RNA from Sigma (St. Louis, MO, U.S.A.), yeast tRNA from the Institute of Organic Chemistry (Siberian Branch of the Academy of Science, U.S.S.R.), calf thymus DNA from Serva, *p*-toluenesulphonylchloride and iminodiacetic acid from Merck (F.R.G.). The polyacrylamide gel electrophoresis kit was obtained from Serva.

Exonuclease A5 (E.C. 3.1.4.1) was isolated from a crude preparation (NPO Biolar, Olaine, U.S.S.R.). Semipure enzyme was obtained by ammonium sulphate precipitation followed by desalting on a Sephadex G-75 column[4].

Protein was quantified according to Lowry using bovine serum albumin as standard. Nuclease, phosphatase and 5'-nucleotidase activities were measured as described[4,5] using as substrates total yeast RNA, *p*-nitrophenyl phosphate and guanosine 5'-monophosphate, respectively. The semipure exonuclease A5 preparation contained about 200 μg 5'-nucleotidase activities were 280, 0.12 and 0.8 U/ml, respectively. Nuclease and phosphatase activities in the presence of cations were determined as shown above but the enzyme solution was preincubated with the corresponding cations for 30 min at 20°C.

Endonuclease (E.C. 3.1.4.9) was isolated from a culture medium containing the bacterium *Serratia marcescens* (pigmentless strain 24, KGU; Department of Microbiology, Kazan, U.S.S.R.) grown according to ref. 6. The endonuclease activity was estimated as follows. To 0.9 ml of $9 \cdot 10^{-2}$ *M* Tris–HCl, pH 8.5, containing $7.7 \cdot 10^{-4}$ *M* magnesium chloride and 1.1 mg of yeast tRNA, 0.1 ml of enzyme solution was added. The mixture was incubated for 15 min at 37°C after which the reaction was terminated by addition of 1 ml of 4% perchloric acid solution. The mixture was kept for 5 min at 0°C, then clarified by centrifugation and the optical density at 260 nm was measured. The quantity of the enzyme added was chosen so that the final substrate conversion did not exceed 10%. The unit of enzyme activity was the quantity of endonuclease which under these conditions causes 1 optical unit increase in optical density of acid-soluble products after 1 h of incubation (U/ml). The specific activity was defined as the ratio of the number of activity units in 1 ml of the solution tested to the optical density of the same solution at 280 nm, U/A_{280}. The endonuclease activity for DNA was measured similarly except that calf thymus DNA was used as a substrate.

The optical density of solutions was measured on a SF-16 spectrophotometer

(LOMO, U.S.S.R.) and on a spectrocolorimeter Specol (G.D.R.). The chromatographic effluent was monitored with a Uvicord II absorbance monitor (LKB, Sweden). Linear gradients were formed with an Ultrograd apparatus (LKB).

Electrophoresis was done on 9% polyacrylamide gel (PAAG) 1 mm-thick vertical slabs at 24 mA using an apparatus made in Tallin (U.S.S.R.). The samples were first dialyzed for 12 h against water, containing 10^{-3} *M* Mg^{2+}, and then lyophilized. To measure the nuclease activity in PAAG according to ref. 7, we used 1% agarose gels prepared in 5 m*M* Tris–HCl, pH 8.5, 1 m*M* Mg^{2+} and 0.2% RNA (or DNA). After blotting of the PAAG onto the agarose gel for 10 min at room temperature, the agarose was soaked in an ethidium bromide solution (2 mg/ml water) and photographed under UV light, the PAAG being stained with Coomassie G-250. For measuring protease activity, a 1% agarose gel was prepapred in 0.1 *M* phosphate buffer containing 0.2% casein. In this case, after blotting the PAAG onto the agarose gel for 30 min at room temperature, the agarose was soaked in 4% trichloroacetic acid, the PAAG being stained as above. A transparent spot on a turbid white background corresponded to the position of the protease. To measure phosphatase activity, the PAAG was submerged in 0.02 *M* *p*-nitrophenylphosphate in an acetate buffer, pH 6.5, containing 0.2% calcium chloride[8] and soaked for 16 h. White opalescent calcium phosphate bands on a transparent yellow background corresponded to the position of the phosphatase. PAAGs stained with Coomassie G-250 were scanned with an Hitachi 557 spectrophotometer (Japan) at 750 nm, the slit width being 0.2 × 6 mm.

The number of iminodiacetic acid groups on chelating sorbents was determined by back titration. To measure their capacities for Cu^{2+}, the sorbents were treated with a 5 mg/ml copper chloride solution for 10 min, and then washed with water and 0.1 *M* acetate buffer, pH 4.0. The bound Cu^{2+} was removed with 0.05 *M* hydrochloric acid, and concentrated ammonia was added to the solution to give a pH of 10. The content of Cu^{2+} was determined by measuring the optical density of the copper tetraammine formed and by comparison with a calibration curve.

Iminodiacetic acid dimethyl ester hydrochloride

A 20-g (0.15 mol) amount of iminodiacetic acid was suspended in 300 ml anhydrous methanol cooled to 0°C, and thionyl chloride (20 ml, 0.28 mol) was slowly added (15 min) with stirring. The mixture was kept at 0°C for 2 h and then boiled for 1 h until the acid was completely dissolved. White needle-shaped crystals were recrystallized from methanol. The yield and melting point of the crystals were 9.7 g (33%) and 182–183°C (methanol), respectively.

Iminodiacetic acid-Silochrom

This sorbent was obtained by three methods.

Method 1. To 15 g of Silochrom in 250 ml toluene was added 10 ml 3-(2',3'-epoxypropoxy)propyltrimethoxysilane. The suspension was boiled for 5 h with stirring, and then transferred onto a porous filter. The sorbent was washed on the filter with 150 ml toluene, 200 ml acetone and dried. This yielded epoxysilochrom containing 4.3% carbon. To 5 g of this sorbent in 30 ml 0.4 *M* sodium hydroxide, 3 g (22.5 mmol) of iminodiacetic acid were added, the pH was adjusted to 10.5 with 4 *M* sodium hydroxide and the mixture was stirred for 1.5 h at 50°C. The sorbent

obtained was washed on a porous filter with 500 ml water, 100 ml acetone and finally dried.

Method 2. To 30 ml anhydrous methanol containing 3.75 g (18.8 mmol) of iminodiacetic acid dimethyl ester were added 5 g of epoxysilochrom (see Method 1). The mixture was stirred at 20°C for 16 h, and the sorbent obtained was washed on a porous filter with 200 ml methanol and 200 ml water. It was resuspended in 30 ml of a saturated copper acetate solution, and kept at 50°C for 1 h. The suspension was washed on a filter with 200 ml water, 100 ml of 0.5 *M* hydrochloric acid, 200 ml water, 100 ml acetone and then dried.

Method 3. To 3 g of epoxysilochrom (see Method 1) were added 100 ml 0.1 *M* sulphuric acid. The suspension was kept for 1h, then washed on porous filter with 300 ml water, 50 ml acetone and dried at a pressure of 0.1 mmHg. The resulting silochrom-diol was resuspended in 15 ml anhydrous dioxane, 0.57 g (3 mmol) of toxyl chloride and 0.57 ml pyridine were added and the mixture was stirred at 20°C for 1 h. The activated sorbent obtained (containing up to 200 μmol tosyl groups per 1 g of the sorbent) was washed on a porous filter with 200 ml dioxane and resuspended in 15 ml dioxane to which 0.6 g (4.5 mmol) of iminodiacetic acid and 1.2 ml pyridine were added. The suspension was stirred at 20°C for 24 h and washed on a filter with 200 ml water, 100 ml 0.5 *M* hydrochloric acid, 200 ml water, 100 ml acetone and then dried.

Iminodiacetic acid-TSK-gel Toyopearl HW-55

Method 1. To 1.3 g of wet TSK-Gel HW-55 were added 1 ml of 15 *M* sodium hydroxide and 2.5 ml epichlorohydrin, and the mixture was stirred at 50°C for 2 h. The activated gel obtained was washed on a porous filter with 200 ml water, and resuspended in 4 ml of 0.4 *M* sodium hydroxide. A 0.2-g (1.5 mmol) amount of iminodiacetic acid was added, the pH was adjusted to 11.7 with 4 *M* sodium hydroxide and the suspension was stirred at 20°C for 20 h. The sorbent obtained was washed on a porous filter with 500 ml water and stored as an aqueous suspension.

Method 2. To suspension of 1.5 g (7.5 mmol) iminodiacetic acid dimethyl ester hydrochloride in 10 ml anhydrous dimethylformamide were added 2.4 ml of 3 *M* sodium methoxide in methanol (7.2 mmol), dropwise while stirring. The solution was filtered, and 1.4 ml (10 mmol) triethylamine were added. To thus solution, 5 g of the wet epoxy-activated gel (see Method 1) were added and mixture stirred for 24–140 h. The sorbent was washed on a porous filter with 500 ml water, treated with 0.05 *M* sulphuric acid for 1 h to open unreacted epoxy groups and finally washed with water to pH 7. The concentration of chelating groups varied from 10 to 110 μmol per 1 ml of the gel depending upon the duration of the reaction.

Sorbents with iminodiacetic acid based on Spheron and cellulose were obtained similarly.

Iminodimethylphosphonic acid-TSK-gel Toyopearl HW 55

To 1.3 g of the epoxy-activated TSK-gel (see Method 1) were added 0.2 g (1 mmol) of iminodi(methylphosphonic acid), the pH was adjusted to 11.7 with 4 *M* sodium hydroxide and the mixture was stirred at 20°C for 20 h. The sorbent obtained was washed on a porous filter with 500 ml water and stored as an aqueous suspension.

RESULTS AND DISCUSSION

The choice of the support and the method of immobilization of stationary ligands determines to a large extent the quality of the sorbent for ligand-exchange chromatography. We used successfully employed by us in the synthesis of sorbents for affinity chromatography of nuclease[4]. The non-specific sorption of proteins, mostly due to silanol groups present on the surface of Silochrom, was reduced by treatment with 3-(2-',3'-epoxypropoxy)propyltrimethoxysilane which leads to the formation of hydrophilic polycondensation products on the surface. The presence of such products is indidated by the appearance of carbon in the sorbents, amounting to more than 4%[4].

The addition of iminodiacetic groups to Silochrom was done in three ways. A satisfactory capacity of the chelating sorbents in terms of the stationary ligand content was not obtained with Method 1 because the addition of the imino groups to the oxirane rings is optimal within the interval of pH 11–12 at which the silica support is not stable. During the synthesis of a chelating sorbent by this method, a side reaction of the oxirane rings with the free carboxylic groups can proceed with the formation of foreign ligands on the sorbent which bind metal ions only weakly. As a rule, weakly bonded metal ions could be eluted from a chelating sorbent with 0.1 *M* acetate buffer, pH 4.0. The use of Method 2 gives an almost monofunctional sorbent because it involves iminodiacetic acid dimethyl ester and methanol as a reaction medium. The chelating sorbents thus obtained were then treated with copper acetate under mild conditions because the hydrolysis of the ester groups under strongly alkaline conditions, as conventionally used, causes dissolution of the silica carrier. Method 3 is based on opening the oxirane rings in acidic solutions followed by activation of the hydroxyl groups with tosyl chloride and addition of iminodiacetic acid. This method has the advantages of simple spectrophotometric monitoring of the activation with tosyl chloride and of the possibility of controlling the extent of activation by varying the quantity of tosyl chloride added.

We also synthesized sorbents with the iminodiacetate groups attached to an hydrophilic organic support, TSK-Gel Toyopearl HW 55, to an organic support, Spheron, and to cellulose. These sorbents are stable at pH 1–14 and are characterized by low non-specific sorption of proteins and metal ions. Essentially monofunctional sorbents containing less than 2% non-specificially bonded ligands were obtained using iminodiacetic acid dimethyl ester (Method 2). The low content of the non-specifically bound ligands was confirmed by the absence of copper ions from eluates in 0.1 *M* acetate buffer, pH 4.0.

For studies with some metal-containing enzymes, we synthesized also chelating sorbents with iminodimethylphosphonic groups having a lower affinity for magnesium and zinc ions, as compared with iminodiacetic acid, but still strongly chelating copper ions.

To verify the usefulness of the synthesized chelating sorbents, we have chosen a preparation of exonuclease A5 actinomyces and bacterial endonuclease from *S. marcescens*. The exonuclease A5 preparation contained non-specific phosphatase and 5'-nucleotidase as the major undesirable contaminants. The affinities of exonuclease, phosphatase and 5'-nucleotidase for iminodiacetate chelating sorbents charged with Cu^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} lay in the following order: phosphatase > nuclease ≫

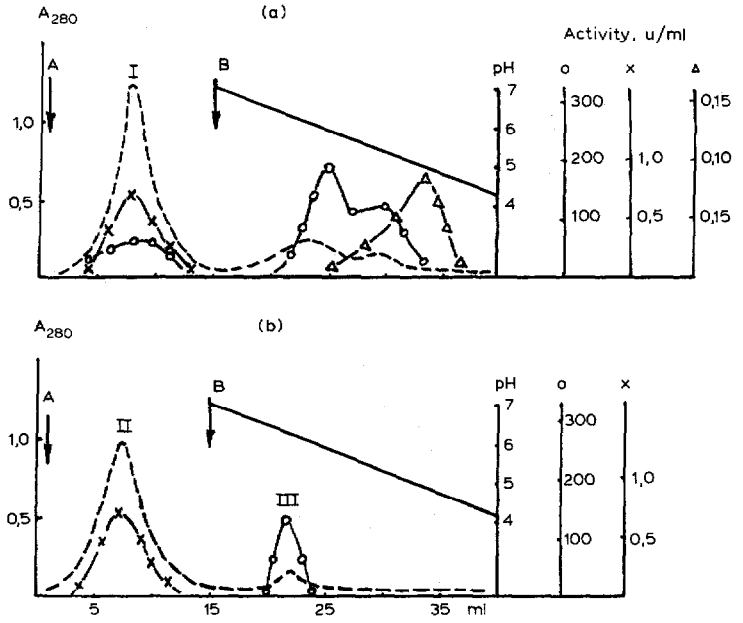

Fig. 1. Ligand-exchange chromatography of an exonuclease A5 preparation (1 mg of protein) on a 2 × 0.8 cm column of a support carrying iminodiacetic groups (36 μmol per ml gel) in nickel form. Elution rate: 10 ml/h. (a) The first purification; (b) rechromatography of peak I (panel a). A, Loading of the sample and start of elution with 5 m*M* Tris–HCl, 1 *M* sodium chloride (solution 1); B, start of a linear gradient of solution 1 to solution 2 (5 m*M* ammonium acetate, 1 *M* sodium chloride, pH 4.2). The nuclease (O—O), 5'-nucleotidase (×—×), and phosphatase (△—△) activities (U/ml) and the protein level (---) were monitored.

5'-nucleotidase. The affinity of phosphatase for a chelating sorbent in manganse form is so high that the elution is possible only under denaturating conditions at pH < 4 or after addition of 10 m*M* EDTA to the eluent. Nickel ions had little effect on the nuclease activity. Therefore the exonuclease was chromatographed on a chelating sorbent in nickel form (Fig. 1).

The semipure exonuclease A5 preparation was subjected to electrophoresis on PAAG. Four major protein bands (Fig. 2a) were revealed of which band 1 has nu-

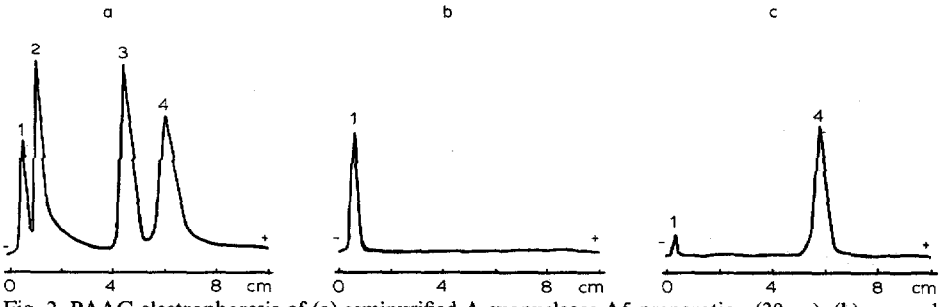

Fig. 2. PAAG electrophoresis of (a) semipurified A exonuclease A5 preparation (30 μg), (b) exonuclease A5 twice purified on a chelating sorbent (10 μg, fraction III in Fig. 1b) and (c) protein of fraction II in Fig. 1b (20 μg).

clease activity, and bands 3 and 4 correspond to phosphatase and 5′-nucleotidase, respectively.

Exonuclease A5 was purified in two stages on a Ni^{2+}–iminodiacetic sorbent. In the first stage the sorbent fully removed the phosphatase, whereas 5′-nucleotidase was not retained (fraction I in Fig. 1a). This fraction, which also contains 25% of total nuclease, was rechromatographed on the same support (Fig. 1b). As fraction I was devoid of proteins with high affinity for the chelating sorbent, the latter was able to retain lower affinity nuclease (Fig. 1b, peak III). Therefore unadsorbed fraction II (Fig. 1b) contained more than 90% 5′-nucleotidase (Fig. 2c).

The use of a pH gradient enabled us to obtain practically homogeneous nuclease, as judged from PAAG electrophoresis (Fig. 2b), containing no additional activities.

Since ligand-exchange chromatography has the advantage of being effective at high salt concentrations, we applied iminodiacetate chelating sorbents to the isolation of *S. marcescens* endonuclease obtained directly from the culture medium. In previous experiments we had shown that the enzyme was adsorbed solely by the sorbents in copper form, not on Ni^{2+}, Co^{2+} and Fe^{3+}. The conditions for ligand-exchange chromatography were optimized for both the sorption (including the pH of the loading solution and the loading rate) and desorption of the endonuclease. We used a 10-ml column of a TSK-Gel HW-55 support with 36 μmol of iminodiacetic groups per 1 ml sorbent (Fig. 3). After being charged with Cu^{2+}, the support was washed with 0.1 *M* sodium acetate buffer, pH 4.0, 0.5 *M* sodium chloride and equilibrated with the same buffer at pH 6.5. Then 4.5 ml of the culture medium (pH 6.5, endonuclease activity 44 000 U/ml, U/A_{280} 4000) were loaded onto the column at the rate of 10 ml/h. Under these conditions the endonuclease present in the culture medium was virtually quantitatively bound to the Cu^{2+}-charged chelating sorbent. The endonuclease was desorbed in a yield of 84% by lowering the pH of the buffer to 5. The recovered enzyme had an activity of 41 000 U/ml and a specific activity, U/A_{280}, of 80 000.

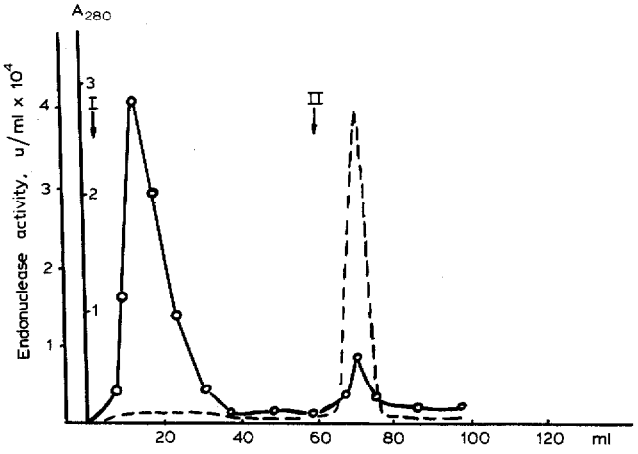

Fig. 3. Ligand-exchange chromatography of the culture medium of a *S. marcescens* on a 6 × 1.8 cm column of a support carrying iminodiacetic groups (36 μmol/ml gel) in copper form. Fraction volume: 10 ml. O—O, Protein content, A_{280}; ---, endonuclease activity, U/ml · 10^4.

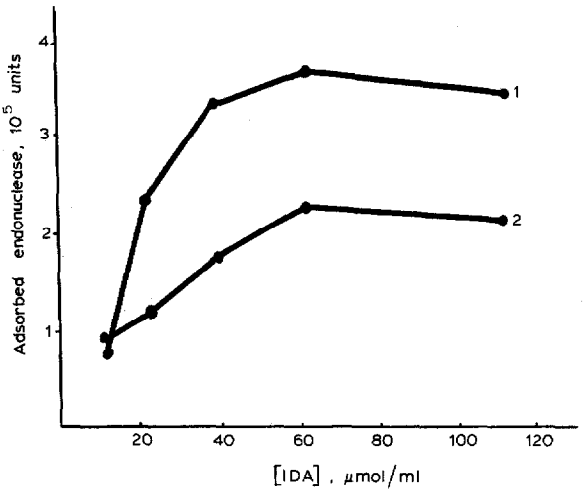

Fig. 4. Capacities of chelating sorbents for endonuclease as a function of the iminodiacetic acid (IDA) residue concentration. (1) Adsorbed endonuclease quantified under static conditions; (2) adsorbed endonuclease quantified by loading of the culture medium onto the column of chelating support.

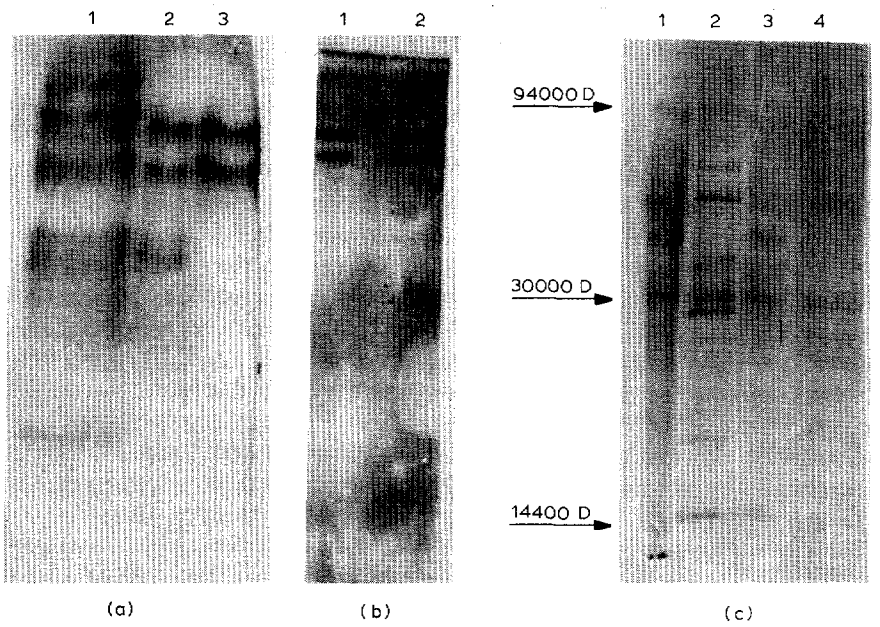

Fig. 5. Electrophoresis of endonuclease *S. marcescens*. (a) Non-denaturing PAAG electrophoresis: 1 = culture medium; 2 = commercial sample, type "B"; 3 = ligand-exchange purified fraction. (b) Detection of nuclease activity in agarose gel: 1 = culture medium; 2 = ligand-exchange purified fraction. (c) Sodium dodecyl sulphate PAAG electrophoresis: 1 = low-molecular-weight calibration kit (Pharmacia); 2 = culture medium; 3 = commercial sample, type "B"; 4 = ligand-exchange purified fraction.

To study the contribution of the stationary ligand concentration to the effectiveness of the endonuclease binding, we prepared sorbents with different concentrations of iminodiacetic groups attached to a TSK-Gel support. To 1 ml of Cu^{2+}-chelating sorbents having different concentrations of the stationary ligand in the range 10.5–111.5 μmol/ml of the gel were added 8-ml portions of the culture medium (pH 6.5, endonuclease activity 33 000 U/ml). The suspensions of the sorbents in the culture medium were shaken for 1 h, and then the adsorbed endonuclease was quantitated. The capacities of the chelating sorbents in copper form were also determined under flow conditions. For this purpose, 3-ml portions of the culture medium (pH 6.5, 120 000 U/ml) were loaded at a rate of 10 ml/h to columns each containing 1 ml of the gel. The columns were eluted with sodium acetate buffer, pH 6.5, 0.5 *M* sodium chloride until the absorbance at 280 nm diminished to zero. The endonuclease activity in the eluate was determined, and the difference between the initial activity and the activity in the eluate was used to calculate the quantity of the enzyme adsorbed. Fig. 4 shows that the chelating sorbents with more than 36 μmol per 1 ml of iminodiacetic groups are most suitable for purification of the endonuclease, while the capacities of those with lower concentrations of the stationary ligand are insufficient.

The preparation of endonuclease *S. marcescens* purified by ligand-exchange chromatography was characterized by PAAG disc electrophoresis at pH 8.3 under non-denaturing conditions (Fig. 5a). The nuclease, protease and phosphatase activities were measured directly in the PAAG. Both the initial culture medium and the preparation obtained contained two equally intense protein bands having nuclease activities for both DNA and RNA (Fig. 5b). The existence of endonuclease *S. marcescens* in two isoforms had been demonstrated earlier[9]. The culture medium also contained protease and phosphatase activities absent from the preparation obtained after chromatography. Scanning of the gel with the electrophoretically separated endonuclease revealed the endonuclease content to exceed 75%. The purified enzyme gave a protein band with a molecular weight of 30 000 as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Fig. 5c).

Thus we have shown that the chelating sorbents with iminodiacetic groups in nickel and copper forms are effective in the purification of exonuclease A5 actinomyces and endonuclease *S. marcescens*.

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