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# PURIFICATION AND CHARACTERIZATION OF CARBOXYLESTERASES OF GREEN BEANS (*PHASEOLUS VULGARIS*)*

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**Abstract**—Phenyl propionate hydrolyzing carboxylesterases (carboxylic ester hydrolase, E.C. 3.1 1 1) in protamine sulfate treated aqueous extract of green beans (*Phaseolus vulgaris* L.) were separated into three fractions by gel filtration on Sephadex G-100. A total of eight fractions were obtained on further purification by CM- or DEAE-cellulose column chromatography. Polyacrylamide gel electrophoresis revealed that none of the fractions was homogeneous. Variations in substrate and inhibitor specificities were demonstrated between the eight fractions Possible structures for the isozymes of these carboxylesterases are discussed.

## INTRODUCTION

CARBOXYLESTERASES (carboxylic ester hydrolases, E.C. 3.1.1.1) of peas (*Pisum sativum* L.) have been purified and separated into three components, of which two were shown to be isozymes.[1] In the previous paper[2] 14 carboxylesterase components were demonstrated in the green bean (*Phaseolus vulgaris* L ). Purified preparations of these components are necessary to study their properties in detail.

The purpose of this study was to fractionate the phenyl propionate hydrolyzing carboxylesterases of the green bean, using column chromatography on Sephadex G-100 and CM- and DEAE-cellulose

## RESULTS

Preliminary observations indicated that 90 per cent of the phenyl propionate hydrolyzing activity of green beans remained after storage of the extract for 46 hr at 4° between pH 5 and 8 Therefore, these esterases appeared to have sufficient stability to withstand purification

### *Purification*

A flow diagram for the fractionation of green bean esterases is shown in Fig. 1 and the purification data are presented in Table 1. Trials with various concentrations of protamine sulfate revealed that a final concentration of 0·2 per cent resulted in a 50 per cent decrease of 260 nm absorbing material present in the green bean aqueous extract. Protamine sulfate concentrations higher than 0·2 per cent did not improve the purification of green bean esterases. Polyvinylpyrrolidone[3] did not effectively remove the 260 nm absorbing material. These results are similar to those reported with pea carboxylesterases.[1]

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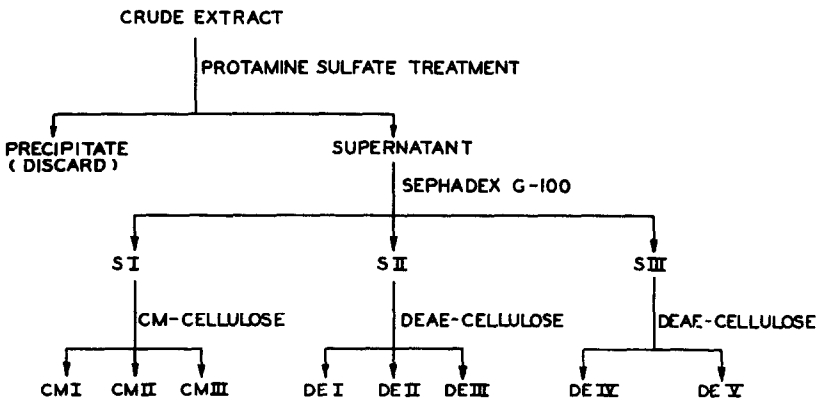


FIG. 1. FLOW DIAGRAM FOR THE FRACTIONATION OF GREEN BEAN CARBOXYLESTERASES

TABLE 1. PURIFICATION OF GREEN BEAN CARBOXYLESTERASES HYDROLYZING PHENYL PROPIONATE

| | Fraction | Vol (ml) | Total units* | Total protein (mg) | Spec. act † (units/mg protein) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| 1 | Crude extract | 60 | 528 | 3024 | 0 17 | | |
| 2 | Protamine sulfate ppt | 65 | 580 | 1930 | 0 30 | 110 | 1·8 |
| 3 | Sephadex G-100 | | | | | | |
| | SI | 63 | 104 | 202 | 0 70 | 19 | 4 |
| | SII | 53 | 53 | 118 | 0 50 | 9 | 3 |
| | SIII | 53 | 195 | 75 | 5 3 | 35 | 34 |
| 4 | CM-cellulose chromatography of SI | | | | | | |
| | CMI | 50 | 19 9 | 18·2 | 8 0 | 35 | 47 |
| | CMII | 43 | 13 | 8 8 | 7·0 | 23 | 41 |
| | CMIII | 44 | 7 3 | 8 1 | 4 8 | 13 | 28 |
| 5 | DEAE-cellulose chromatography of SII | | | | | | |
| | DEI | 44 | 30 6 | 2 5 | 28 9 | 41 | 170 |
| | DEII | 44 | 8 2 | 5 1 | 2 2 | 11 | 12 |
| | DEIII | 66 | 6 0 | 22 9 | 0 3 | 8 | 2 |
| 6 | DEAE-cellulose chromatography of SIII | | | | | | |
| | DEIV | 44 | 35 8 | 8 0 | 5 0 | 9 2 | 30 |
| | DEV | 55 | 33 2 | 9 7 | 4 9 | 8 5 | 29 |

* 1 Activity unit represents 1$\mu$ equivalent of substrate hydrolyzed/min/ml of enzyme solution.
† Most active tube of each fraction.

Gel filtration of protamine sulfate treated extract on Sephadex G-100 separated the esterase activity into three fractions (Fig. 2, A). The three fractions were lyophilized and stored dry at —20° for further investigation. Polyacrylamide-gel electrophoresis (Fig. 3, A–D) of the fractions showed the presence of Group 1 esterases in fraction SI, Group 1 and 2 in SII and Group 3 with some contamination of Group 1 (apparently due to tailing) in SIII. This observation indicates that the pattern of separation on polyacrylamide gel

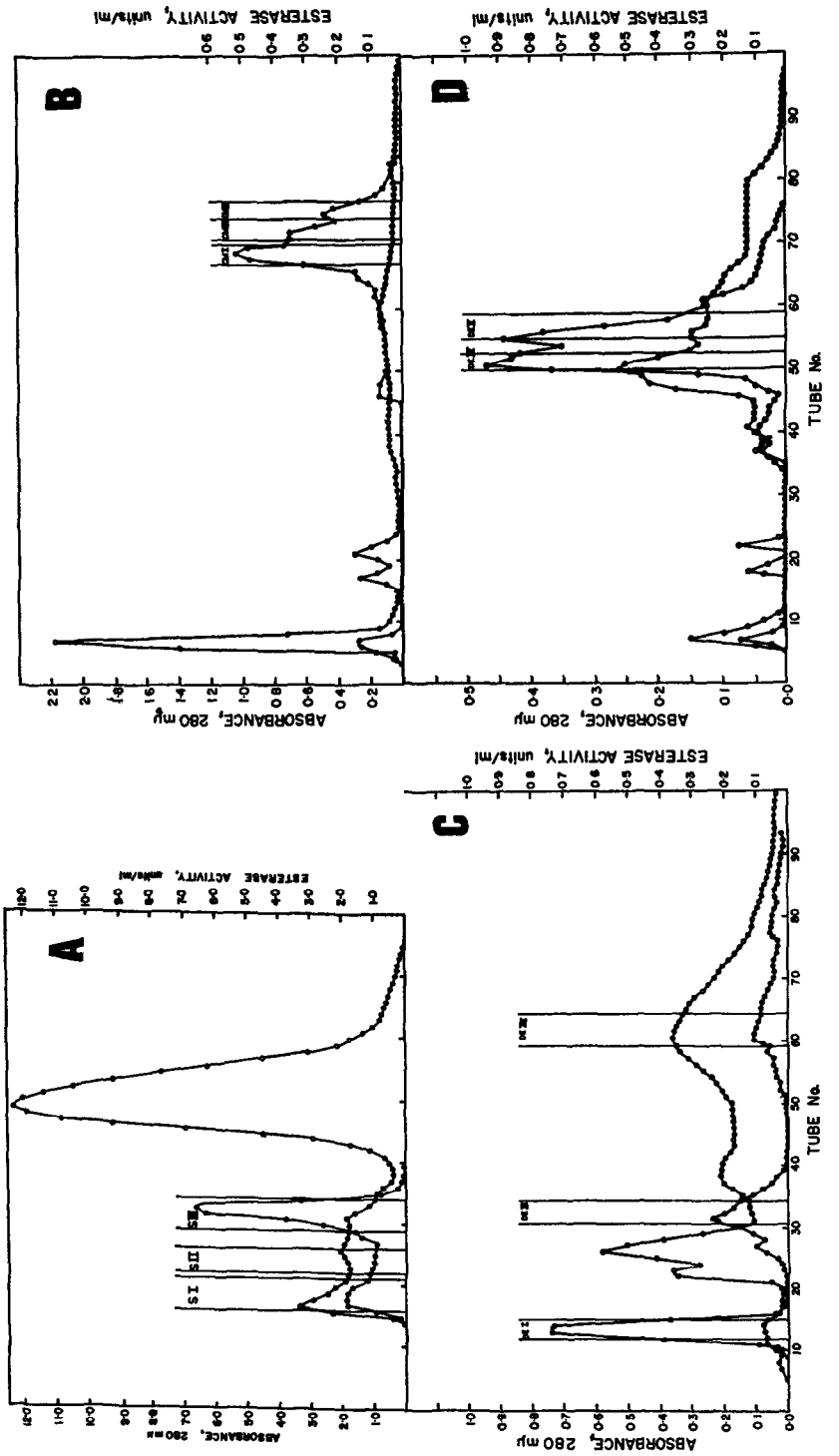


FIG 2. CHROMATOGRAPHY OF GREEN BEAN CARBOXYLESTERASES.

A is the gel filtration of protamine sulfate treated green bean extract on Sephadex G-100. B is the chromatograms of fraction SI on CM-cellulose. C and D are the chromatograms of fractions SII and SIII, respectively, on DEAE-cellulose. See text for details. ●—●—●—●—●, Esterase activity, ○—○—○—○—○—○, absorbance at 280 nm

correlated well with the results of gel filtration with regard to molecular size. That is, Group 1 was eluted first from the Sephadex and showed the slowest migration on polyacrylamide gel, while Groups 2 and 3 were retarded on the Sephadex and migrated faster in the polyacrylamide gel matrix. This would suggest that the relative molecular size was Group 1 > Group 2 > Group 3.

Preliminary attempts to further fractionate the esterase activity of fraction SI on DEAE-cellulose were unsuccessful. Chromatography of fraction SI on CM-cellulose, however, revealed a partial separation of the enzyme activity into three fractions (Fig 2, B). Activity of fraction CMIII was very low and no attempt was made to characterize it further. Fractions CMI and CMII were subjected to electrophoresis on polyacrylamide gel (Fig. 3, E and F). Close examination of the gel revealed three esterase bands in CMI and two in CMII. Band 1 was more active than band 2 in CMII, while CMI contained slight activity from band 3. Absence of bands 4 and 5 from either of these fractions suggests that these might have been present in fraction CMIII.

Esterase activity of fraction SII was separated into three fractions by chromatography on DEAE-cellulose (Fig 2, C). 66 per cent of the activity of fraction SII was in fraction DEI and was purified 170 fold over that of the crude extract Since the activity of fraction DEIII was very low, this fraction was dialyzed against water for 3 days in the cold, lyophilized and redissolved in water. Electrophoretic analysis of these fractions (Fig. 3, H–K) revealed that fraction DEIII contained only group two esterases, while bands 1–3 were in fraction DEI and bands 3, 4 and 5 were in fraction DEII.Thus, band 3 was present in both fractions DEI and DEII, even though the fractions were well separated in the elution pattern. When naphthol AS acetate was the substrate (Fig 3, L–O), Group 2 was shown to contain five distinct bands of esterase activity. Bands 1 and 2 of DEI did not hydrolyze naphthol AS acetate, whereas bands 3 and 4 of DEII did, confirming that these bands have identical substrate specificity as the same bands in the whole green bean extract.[2]

Chromatography of fraction SIII on DEAE-cellulose resulted in the partial separation into two fractions (Fig 2, D). The electrophoresis pattern (Fig. 3, P–S) shows that each fraction contained two bands of the four bands of SIII. Tube 54, which was midway between these fractions, also contained two bands, one each from DEIV and DEV. This suggests that these four esterase bands could be completely separated and exist as independent bands rather than as doublets.

*Substrate Specificity*

The fractions of esterase activity obtained from DEAE-cellulose chromatography were examined for their ability to catalyze the hydrolysis of nine esters. Results (Table 2) reveal that butyryl esters were not hydrolyzed appreciably by any of the fractions except DEIV and DEV. Fractions CMI and CMII showed similar activity with acetyl and propionyl esters of phenol. However, activities differed towards acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate and glycerol. Similar results were obtained with fractions DEI and DEII where the activity towards phenyl acetate and propionate were identical, but DEII showed specificity for the acetyl and propionyl esters of sodium 2-naphthol-6-sulfonate, while DEI preferentially hydrolyzed tripropionin Since these four fractions (CMI, CMII, DEI, and DEII) comprise the Group 1 enzymes (Fig. 3), differences in substrate specificities must exist between the bands of esterase activity in Group 1

Fractions DEIII, which contained Group 2 esterases, and DEV demonstrated a specificity for the propionyl esters. In contrast to the other fractions, fractions DEIV and DEV

TABLE 2 RELATIVE ACTIVITY OF GREEN BEAN CARBOXYLESTERASE FRACTIONS TOWARD VARIOUS SUBSTRATES

| Substrates | Crude extract | CMI | CMII | DEI | DEII | DEIII | DEIV | DEVR |
|---|---|---|---|---|---|---|---|---|
| Triacetin | 13 | 27 | 15 | 9 | 25 | 38 | 33 | 55 |
| Tripropionin | 29 | 21 | 44 | 53 | 12 | 48 | 41 | 82 |
| Tri-*n*-butyrin | 11 | 6 | 9 | 8 | 0 | 0 | 30 | 30 |
| Acetyl ester of sodium 2-naphthol-6-solfonate | 11 | 79 | 38 | 0 | 84 | 16 | 12 | 17 |
| Propionyl ester of sodium 2-naphthol-6-sulfonate | 17 | 140 | 77 | 12 | 85 | 40 | 13 | 39 |
| *n*-Butyryl ester of sodium 2-naphthol-6-sulfonate | 14 | 1 | 2 | 3 | 9 | 6 | 22 | 55 |
| Phenyl acetate | 107 | 125 | 128 | 106 | 107 | 73 | 104 | 66 |
| Phenyl propionate | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Phenyl *n*-butyrate | 59 | 8 | 15 | 16 | 14 | 12 | 75 | 80 |

Activity with phenyl propionate = 100.

revealed considerable activity towards the butyryl esters; however, several differences in substrate specificities are evident.

*Inhibitor Specificity*

Organophosphorus inhibitors and *p*-chloromercuribenzoate (PCMB) have been used to study the esterases of green beans[2,4] and other esterase systems.[1,5–11] Unfortunately sufficient quantities of fraction CMIII and DEIII were not available for these studies. None of the fractions studied showed similar sensitivity towards diethyl *p*-nitrophenyl thiophosphate (parathion) (Fig. 4, A–D) Fraction CMI was more resistant to parathion than CMII. The curves suggest that at least two esterases were present in CMI; one was inhibited between pI (negative log $_{10}$ of the molar inhibitor concentration) 8 and 4, while the other was inhibited between pI 4 and 1. Similarly, two esterases were present in CMII and were inhibited at the same pI ranges. Since CMI and CMII contained only band 1 and 2 of Group 1 (Fig. 3), the sensitive and resistant esterases of the two fractions must be the same. Also since CMII had more of band 1 than CMI, band 1 was the more sensitive component and band 2 the more resistant. However, fraction DEI, which contained bands 1–3, had only the sensitive esterase present (Fig. 4, C) Since all esterases are not detected with α-naphthyl acetate,[12] esterases other than those that appeared on the gel might have caused these fractions of Group 1 esterases to differ in sensitivity to parathion when phenyl propionate was the substrate.

Several inhibition curves in Fig. 4, A–D did not reach 100 per cent suggesting that another esterase, more resistant to parathion, was present. Fraction DEI, being more sensitive to parathion than fraction DEII, exhibited a single sigmoid curve indicating that all three bands

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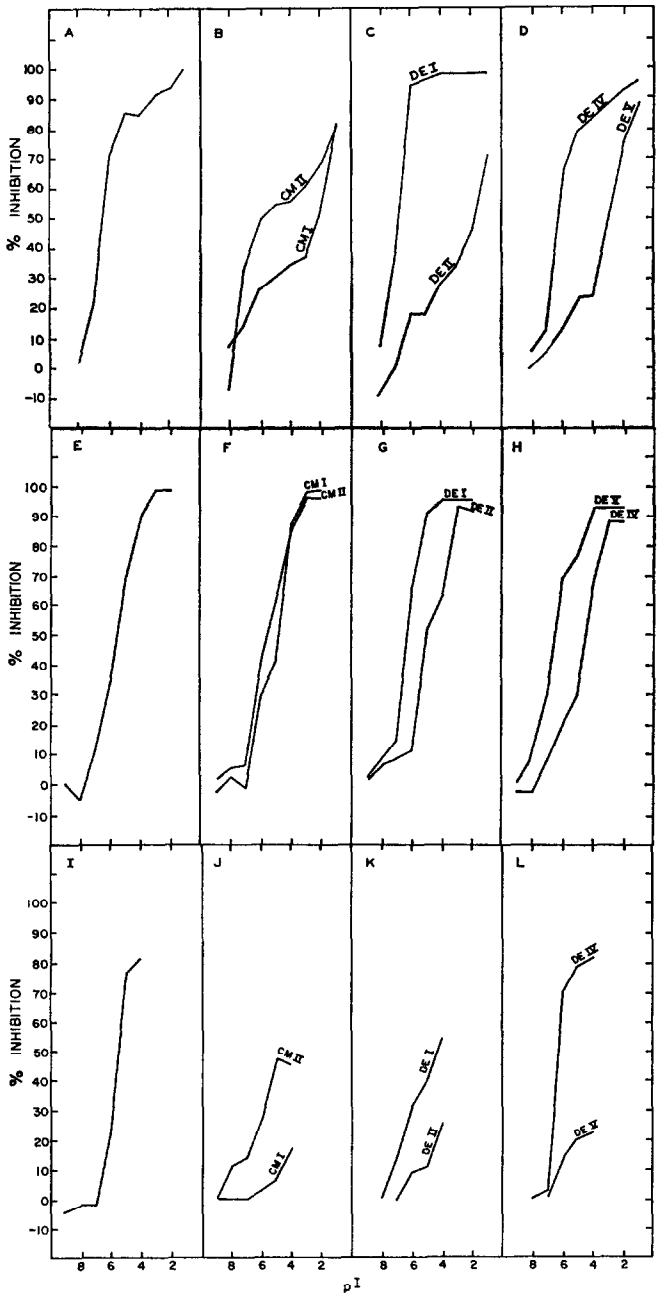


FIG 4 INHIBITION CURVES OF THE HYDROLYSIS OF PHENYL PROPIONATE BY SIX PURIFIED FRACTIONS OF GREEN BEAN CARBOXYLESTERASES WITH PARATHION (A–D), DFP (E–H), AND PCMB (I–L).

A, E and I are curves for the unfractionated green bean extract Percent inhibition is plotted against the negative $\log_{10}$ of the M inhibitor concentration

(1–3) showed similar sensitivity to parathion. The inhibition curve of fraction DEII suggests the presence of esterase varying in sensitivity to parathion. Since band 3 was present in DEI and DEII, band 3 might have been the more sensitive component of DEII and bands 4 and 5 could account for the more parathion resistant activity.

Fractions DEIV and DEV also differed in sensitivity to parathion. In the previous paper[2] all the components of Group 3 showed equal sensitivity to $10^{-4}$ M parathion. However, in Fig. 4, D DEV was inhibited 23 per cent, whereas DEIV was inhibited 83 per cent at $10^{-4}$ M parathion. Therefore, these enzymes may vary in their sensitivity to parathion with different substrates.

Although the fractions varied in their sensitivities to diisopropylphosphorofluoridate (DFP) (Fig. 4, E–H) suggesting the presence of sensitive and resistant components in each fraction, all the fractions were completely inhibited at the highest concentration of inhibitor used. Therefore, esterases in these fractions would be classified as carboxylesterases (carboxylic ester hydrolyases, E.C. 3.1.1.1).[10]

Each fraction differed in its sensitivity to PCMB (Fig. 4, I–L). These results suggest that the purified fractions, as well as the green bean extract, contained sensitive and resistant esterase components. The similarity between the inhibition curves of DEIV and the green bean extract indicates that fraction DEIV contained the major portion of the PCMB sensitive esterase activity

## DISCUSSION

Ecobichon[13] recently described the separation of microsomal carboxylesterases of bovine liver into a rapidly migrating band and a group of four slower moving bands on starch-gel electrophoresis. Gel filtration on Sephadex G-200 demonstrated that these carboxylesterases were of similar molecular size though differing in net electrical charge. In a similar manner, pea carboxylesterases were not fractionated by gel filtration on Sephadex G-100. By contrast, gel filtration on Sephadex G-100 and subsequent electrophoresis suggest that green bean carboxylesterases are not of similar molecular size but consist of three fractions. The order of elution of the fractions from Sephadex G-100 is the precise inverse of the order in which the esterases migrate in polyacrylamide-gel electrophoresis, i e. the relative molecular sizes are in the order Group 1 > Group 2 > Group 3. Furthermore, charge difference must exist between the three groups as well as between the individual bands of esterase activity. Further refinement of the chromatographic procedures may result in a more complete separation of the individual bands.

The five tetrameric isozymes of lactate dehydrogenase have been reported to be composed of two monomers.[14] Desborough and Peloquin,[15] as a result of genetic studies on *Solanum* tubers, suggested that the esterases of this plant were tetramers composed of one, two, or three different types of monomers. These authors believe that the three monomers, when combined in all possible combinations, would yield the 15 esterase isozymes found by starch-gel electrophoresis. Although, this model would account for the approximate number of esterase bands present in green beans, it would not explain the differences in molecular size which were observed. On the other hand, Smithies and Connell[16] suggested that the heptoglobin molecule was made up of two types of subunits, but the total number

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of subunits/molecule was variable, so that separation of heptoglobins into several components by gel electrophoresis or on Sephadex[17] depended on differences in molecular size.

Since the behavior of the green bean esterase isozymes is similar to that of the heptoglobins, these esterase isozymes also may contain a variable number of subunits/molecule. Although further investigations are necessary to determine the exact number and nature of subunits in each isozyme, the results from the inhibitor studies (Fig. 4) suggest that the isozymes of green bean carboxylesterases are composed of two subunits, one resistant and one sensitive. If it is assumed that the more sensitive subunit possessed a lower negative charge than the resistant subunit, then the slower migrating bands of each group would be more sensitive, while the faster migrating bands of each group would be resistant. Such was the case for Groups 1 and 3 for which data are available with the exception that DEV was more sensitive to DFP than DEIV.

## EXPERIMENTAL

Procedures used for the preparation of the green bean extract, substrates, and inhibitors have been described earlier.[4,5] Assay procedure and gel electrophoresis were carried out as detailed in previous papers [1,4,5] Twice distilled water with the final distillation in an all glass apparatus was used throughout this work

### *Protamine Sulfate Treatment*

Protamine sulfate was used for precipitating nucleic acids and their anionic biopolymers.[18] A 2% solution of protamine sulfate (Sigma) was titrated with 1 N NaOH to pH 6 5. 1 ml of this solution was added to 10 ml of the green bean extract, mixed and allowed to stand for 30 min in the cold The precipitate was removed by centrifugation at 15,000 *g* for 10 min

### *Sephadex Chromatography*

Sephadex columns were prepared and developed as previously described [1] 60 ml of enzyme extract (1:5, w/v) were treated with protamine sulfate and lyophilized. The dried material was dissolved in 15 ml of water and applied to the column The eluent was continuously monitored at 280 nm and collected in 10 ml fractions

### *Cellulose Ion-exchange Chromatography*

A microgranular DEAE-cellulose (type DE52, 1 04 mequiv/g) column (2 5 × 40 cm) was prepared in accordance with the instructions from the manufacturer (Whatman Tech Bull IE3) and equilibrated with 1 mM tris-phosphate (pH 7 4). After the chromatographic run, the exchanger was regenerated in the column by eluting with 100 ml of 0 1 M $KH_2PO_4$ followed by 1 mM tris-phosphate (pH 7 4) until the pH of the eluent was 7 4. A column (2 5 × 40 cm) of microgranular CM-cellulose (type CM52, 1 0 mequiv/g) was packed, a constant flow (45 ml/hr) micropump was attached and CM-cellulose was equilibrated with 1 mM phosphate (pH 6 6). Regeneration of the CM-cellulose in the column was accomplished by elution with 100 ml of 0 1 M phosphate (pH 6 6) followed with 1 mM phosphate (pH 6 6) until the pH of the eluent was 6 6

Fractions from Sephadex chromatography were lyophilized, dissolved in 5 ml of water and applied to the desired ion-exchange resin. The column was developed with the appropriate buffer until the first protein fraction appeared and then with a linear gradient to 0 5 N NaCl in the starting buffer 10 ml fractions were collected as the eluent was continuously monitored at 280 nm

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