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GLUCAN PHOSPHORYLASE IN THE LEAVES OF *DENDROPHTHOE FALCATA*: PURIFICATION AND CHARACTERIZATION OF ENZYME

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Abstract—α-Glucan phosphorylase of the leaf tissues of *D. falcata* was partially purified and isolated in two forms, A and B, by DEAE-cellulose column chromatography. Both the forms utilized soluble starch with equal efficiency, the K_m value being 0·12 and 0·25 g/l for A and B respectively. Form A phosphorylase utilized glycogen efficiently, with K_m value of 0·42 g/l but glycogen did not serve as primer for the B form. In contrast, the B form alone could utilize achroic dextrin, though with less efficiency than starch. The K_m values for glucose-1-phosphate were 5 mM and 1·7 mM for A and B. AMP activated phosphorylase A, at the optimum pH, but not the B form. Among other differences between the two enzyme fractions were stability towards heat, linearity of activity with protein concentration and response to added cations and mercaptoethanol. The two enzymes were sensitive to some phenolics; phloridzin, in particular, was highly inhibitory to fraction B, whereas fraction A was inhibited only slightly. The phenolics in the leaves of *D. falcata* were highly inhibitory to both forms of enzyme.

INTRODUCTION

α-GLUCAN phosphorylase has been purified extensively from storage organs such as seeds[1-3] and tubers.[4-6] No detailed study exists on the purification of enzyme from a leaf tissue except for the recent report of the demonstration by gel-electrophoresis of two phosphorylases in leaf extracts of *Spinacia oleracea* and immature cotyledons of *Vicia faba*.[7]

Purification of any enzyme from mistletoe has not yet been reported, probably due to the inherent difficulties in obtaining active cell free preparations. An earlier paper[8] dealt with a technique for avoiding the inhibitory effect of phenolics on enzyme activity and enzyme solubilization. The present communication deals with the purification of α-glucan phosphorylase from the leaves of *D. falcata* and separation into two major isozymic forms with different kinetic properties.

RESULTS

Enrichment of the Enzyme and Freedom from Contaminating Enzymes

The protocol of enzyme purification is summarized in Table 1. Elution of enzyme and of protein from DEAE-cellulose column gave two main peaks. The enzyme was enriched 92- and 51-fold in fraction A and B respectively. Fraction C, a minor peak between fraction A and B, was enriched 2·6-fold.

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TABLE 1. PURIFICATION OF GLUCAN PHOSPHORYLASES

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Fold-enrichment	Recovery (%)
16,000 *g* supernatant (Fraction I)	1500	1380	2205	0·63	—	100
30–60 saturation ammonium sulfate fraction, dialysed and centrifuged clear (Fraction II)	60	914	394	2·32	3·7	66
DEAE-cellulose chromatography*						
Fraction A	80	256	4·4	58·19	92·4	18·5
Fraction B	80	268	8·4	31·90	50·7	19·4
Fraction C	40	90	56 0	1 61	2·6	6·5

* 30 ml fraction II was applied. The data have been calculated for 60 ml solution.

Enzyme fraction A and B were free from the activities of glucose phosphate isomerase and phosphatase action towards glucose-1-phosphate, 5′-AMP, ATP and β-glycerophosphate, when tested under conditions of standard phosphorylase assay.

Linearity of Assays With Protein Concentration

Fraction A enzyme exhibited linearity of activity up to 5·5 μg and fraction B up to 31·5 μg protein. All the following studies were carried out within the range of enzyme linearity.

The Effect of Storage

The two preparations were stable for several days when stored at 2–5° in 0·005 M tris-HCl buffer, pH 7·2. In contrast, the crude extract of the enzyme (Fraction I) was very susceptible to storage, about 75 per cent loss in enzymic activity occurring in 2 days at 2–5°.

The Effect of Temperature

The two enzymes exhibited different heat-stability. Fraction A enzyme was stable up to 50° when heated for 5 min, though raising the temperature to 60° caused marked inactivation (67 per cent). There was a further 15 per cent loss on raising the temperature to 70°. Fraction B enzyme, on the other hand, proved to be more heat labile and even failed to withstand 50° for 5 min, when it lost 73 per cent of its activity. Further raising the temperature to 60° caused only 10 per cent additional loss in activity. A separate experiment wherein the heat-stability was tested in the presence of primer, indicated that the primer did not protect either fraction of enzyme. Fraction A and B had optimum activity at 50 and 45° respectively.

pH Optima

The optimum pH of fraction A and B was determined in citrate and imidazole buffers in the absence and the presence of 4 mM AMP or ATP. In the absence of supplements both the enzymes had the optimum activity at pH 6·0, irrespective of the buffer used. How-

ever, in imidazole buffer, fraction B phosphorylase did not show a sharp optimum, there being a broad optimum in the range 6·0–6·5.

When the standard assay system employing citrate buffer was supplemented with ATP and AMP, a shift in the optimum pH to 6·5 was observed with fraction A phosphorylase. With imidazole buffer, the optimum pH shifted to 7·0 on supplementing with either ATP or AMP. With fraction B enzyme, AMP, but not ATP, caused a shift in the optimum pH 6·5, when citrate buffer was used. The optimum pH remained unaltered on incorporation of ATP or AMP in the presence of imidazole buffer.

The Effect of Increasing Concentration of AMP and ATP

The effect of ATP and AMP used in the concentration range 0·4–8 mM on fraction A activity at pH 6·5 and on fraction B, at pH 6·0 was tested in the presence of citrate buffer (Table 2).

AMP and ATP, at 3 mM concentration, activated the starch phosphorylase activity of fraction A by 46 and 70 per cent respectively. Various concentration of AMP tested had no effect on fraction B activity. ATP produced 96 per cent activation at 6 mM concentration.

TABLE 2. EFFECT OF AMP AND ATP ON GLUCAN PHOSPHORYLASES

Final concentration of nucleotide (mM)	Activity (units/ml)		
	Fraction A		Fraction B
	AMP	ATP	ATP
Nil, control	(2·50)		(2·50)
0·4	3·20	2·65	2·55
1	3·35	2·65	2·55
2	3·40	2·75	2·90
3	3·65	4·25	3·85
6	3·65	4·05	4·90
8	3·70	3·60	4·25

The effect of 5′-AMP and ATP on fraction A was tested at pH 6·5 and ATP on fraction B at pH 6·0. The control values, in the absence of supplements, are recorded in parenthesis.

The Effect of Sugars and Other Metabolites

The sugars and sugar phosphates tested were: galactose, glucose, fructose, maltose, sucrose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, 6-phosphogluconate and were employed in 2 mM concentration, with the exception of glucose which was tested also at 20 mM level. The intermediates tested included 3-phosphoglycerate, phosphopyruvate, pyruvate, oxoglutarate, succinate, malate, glutamate and glycollate and were used in a concentration of 2 mM. The various agents tested did not affect the activity of either enzyme fraction.

The Effect of Cations and Anions and Chelating Agents

There were quantitative differences in the response by the two enzyme fractions towards cations (Table 3).

TABLE 3. EFFECT OF CATIONS AND ANIONS ON GLUCAN PHOSPHORYLASES

Additive†	Final concentration (mM)	Fraction A*		Fraction B	
		Activity (units/ml)	Activation (+) or inhibition (−) (%)	Activity (units/ml)	Activation (+) or inhibition (−) (%)
None, control		2·10	—	2·20	—
$AgNO_3$	2	0·88	−58	1·08	−51
$FeSO_4$	2	2·29	+9	2·71	+23
$HgCl_2$	0·4	0·36	−83	0·92	−58
$ZnSO_4$	2	2·24	—	2·10	—
	4	1·74	−17	1·96	−11
$(NH_4)_6\ Mo_7\ O_{24}$	2	1·68	−20	2·10	—

* The protein concentration of the two fractions was equalized by supplementing the assay system of fraction A with externally added bovine serum albumin.

† Addition of $CaCl_2$, $CoCl_2$, $CuCl_2$, $Fe_2\ (SO_4)_3$, $MnCl_2$, $MnSO_4$, $NaNO_3$ or NH_4Cl (2,4 or 5 mM), $MgSO_4$ (0 01–40 mM), Na F (20 mM) or $KH_2\ PO_4$ (0·2 mM) did not affect either fraction.

Hg^{2+} proved more inhibitory to form A than B, the former being inhibited by 83 and the latter 58 per cent. Ammonium molybdate caused 20 per cent lowering of fraction A activity, leaving fraction B unaffected. On the other hand, Fe^{2+} activated fraction B phosphorylase by 23 per cent, whereas the activity of A fraction was enhanced only 9 per cent.

EDTA at concentration of 1 and 2 mM did not affect the activity of the two enzymes. CN^-, on the other hand, led to 31 and 47 per cent inhibition respectively at concentration of 1 and 4 mM, and two forms of enzyme being equally inhibited.

Effect of Sulfhydryl Compounds and their Binding Agent and Other Reducing Agents

Glutathione (2 mM) and cysteine (0·1–10 mM) did not affect the two activities. Mercaptoethanol too, had no effect on Fraction A at the three concentrations tested (0·2, 2 and 4 mM). However, the last two concentrations resulted in about 25 per cent inhibition of fraction B activity.

The sulfhydryl binding agent, *p*-chloromercuribenzoate, led to progressively increased inhibition of the two enzymes with increasing concentrations. On preincubation with 4 mM *p*-CMB for 60 min, fraction A was inhibited 62 per cent and fraction B 75 per cent. Attempts to reverse the inhibition by dialysis of inhibited enzyme against β-mercaptoethanol, or by the addition of cysteine to the inhibited enzyme, did not succeed.

Ascorbic acid, sodium bisulfate and sodium dithionite (at a final concentration of 20 mM) caused, respectively, 18 and 21, 79 and 80 and 80 and 87 per cent inhibition of fraction A and B activities.

Effect of Phenolic Compounds

In many cases the two enzyme fractions exhibited quantitatively different responses towards phenols. Simple phenols such as phenol, tyrosine and ferulic acid, proved to be non-inhibitory to either fraction. Others, such as *p*-coumaric acid and vanillin, were slightly inhibitory to fraction B, the activity of fraction A remaining unaltered (Table 4).

TABLE 4. EFFECT OF PHENOLIC COMPOUNDS ON GLUCAN PHOSPHORYLASES

Additive †	Final concentration (mM)	Fraction A		Fraction B	
		Activity (units/ml)	Activation (+) or inhibition (−) (%)	Activity (units/ml)	Activation (+) or inhibition (−) (%)
None, control		3·20		3·35	
Coumarin	20	2·75	−14	3·00	−10
p-Coumaric acid	20	3·20	—	2·50	−25
Vanillin	20	3·15	—	3·00	−10
Resorcinol	20	3·15	—	1·60	−52
Catechol	20	4·22	+32	3·25	—
Caffeic acid	10	1·60	−50	2·35	−30
Phloroglucinol	20	3·20	—	1·45	−57
Phloridzin	10	2·70	−16	1·05	−69
Tannic acid*		0·86	−73	1·44	−57

The final pH of the additive was adjusted to neutrality. The protein concentration of the two fractions was equalized by supplementing the assay system of fraction A with externally added bovine serum albumin. Symbol (−) denotes that no effect was encountered at the concentration tested.

* 50 μg tannic acid was employed.

† Addition of phenol (20 mM), tyrosine (2 mM), cinnamic acid (20 mM) or ferulic acid (10 mM) had no effect on either fraction at the concentration tested.

Cinnamic acid failed to produce any effect on the enzymic activities. Coumarin was slightly inhibitory to both the forms. Of special interest was the different effect on the two enzymes of resorcinol and phloroglucinol. These produced respectively 52 and 57 per cent inactivation of fraction B phosphorylase, but activity of the A fraction remained unaffected. Catechol, however, was non-inhibitory for B and actually activated fraction A. Caffeic acid, another dihydroxyphenol, inhibited the A and B fraction by 50 and 30 per cent respectively. Tannic acid led to 73 and 57 per cent inhibition of fraction A and B respectively, indicating the more sensitive nature of the fraction A. Of special interest was the high sensitivity of B fraction towards phloridzin, in contrast with the weak response by fraction A.

The phenolics derived from *Cuscuta* species were non-inhibitory. Phenolics from *D. falcata* elicited 57 and 60 per cent inhibition of A and B fractions respectively. *O. cernua* phenolics caused inhibition of fraction *A* phosphorylase (28 per cent), whereas fraction B enzyme was unaffected.

Substrate Specificity

For both the forms, glucose-1-phosphate could not be replaced in assay system by glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate, used in equivalent concentration.

Primer Specificity

It was of interest to examine whether the two fractions exhibited any differences in the primer specificities and also if starch isolated from different sources could be utilized differently by the two fractions. The results are recorded in Table 5.

With both the fractions, 10–25 per cent activity was observed even in the absence of added primer. Besides soluble starch, the two fractions could utilize white dextrin, amylopectin and amylose as primers, though with differing efficiences. As compared to soluble starch, dextrin though equally active for B, was only 35 per cent active with A. Amylopectin was as active as soluble starch for B, but more active than soluble starch for A. A significant distinction between the two enzymes was that whereas fraction A enzyme could use glycogen but not achroic dextrin, the reverse was true for fraction B phosphorylase. Maltose was ineffective when tested in amounts ranging from 0·25 to 6·0 mg per assay system.

TABLE 5. PHOSPHORYLASE ACTIVITY USING DIFFERENT PRIMERS

Primer	Activity (units/ml)	
	Fraction A	Fraction B
Soluble potato starch	2·15	1·90
Achroic dextrin	Nil	1 45
Amylopectin (from potato starch)	2·95	2·00
Amylose	2 25	1·45
Dextrin (White)	0·75	1·85
Glycogen (oyster)	2 10	Nil
Maltose	Nil	Nil
Starch from:		
Cuscuta reflexa (filaments)	1·95	1·80
Orobanche cernua (whole growth)	2·65	1·60
Oryza sativa	2·30	1·70
Phaseolus vulgaris	2·35	0 75
Zea mays	2·55	1·75

Assay system contained 1 mg each of the primers in the soluble or suspension form. The activity without primer, 0·45 and 0·40 units/ml respectively for Fractions A and B, was subtracted.

On testing the priming capacities of native starch from different sources it was found that starch prepared from the whole growth of *O. cernua* was most efficiently utilized as primer by fraction *A*. Starch from *P. vulgaris* served as a better primer compared to *C. reflexa*. With fraction *B*, on the other hand, starch from *C. reflexa* was significantly more reactive than that from *P. vulgaris*.

The Effect of Primer and Substrate Concentration

The Michaelis–Menten constant (K_m) values evaluated from Lineweaver–Burk plots in citrate buffer, pH 6·2, for soluble starch were respectively 0·12 and 0·25 g/l. for fraction A and B. The V_{max} of the two fractions, however, was different; the respective values for A and B were 40 and 22·7 units/mg protein. The K_m value for glycogen was 0·42 g/l. and V_{max} 101 units/mg protein for fraction A phosphorylase.

The substrate saturation curve with glucose-1-phosphate exhibited normal hyperbolic response with either fraction. The K_m values for the fraction A and B were 5 mM and 1·7 mM respectively. The V_{max} values for fraction A and B were 77 and 40 units/mg protein respectively.

DISCUSSION

At least two types of phosphorylase are present in leaves of *D. falcata*. These phosphorylases are separable by DEAE-cellulose chromatography and are distinguishable by several properties.

The two phosphorylases had different heat-stability characteristics and optimum temperature for activity. Fe^{2+} activated fraction B more significantly than fraction A; molybdate inhibited fraction A without influencing fraction B. In general, fraction A enzyme was more sensitive than fraction B enzyme to the inhibitory action of cations. The two enzyme fractions differed with respect to the action of high concentrations of mercaptoethanol; the B form was inhibited, whereas the A form was unaffected.

Di- and tri-hydroxyphenols elicited different responses towards the two enzymes. Whereas, *p*-coumaric acid, resorcinol and phloroglucinol were powerful inhibitors of fraction B phosphorylase, fraction A activity remained unaltered. Very recently Black and Sondheimer[9] studied the influence of polyphenols on potato phosphorylase and suggested that *o*-dihydric phenols exerted inhibitory effect due to the presence of *o*-diphenol oxidase in the preparation which could oxidize chlorogenic acid to *o*-quinone. In view of the fact that potato phosphorylase is considered a 'SH enzyme'[6] they suggested that the inhibition caused by oxidized chlorogenic acid was due to the addition of the *o*-quinone of chlorogenic acid to one or more sulfhydryl groups of the phosphorylase. Such a mechanism seems to be unlikely in the case of phosphorylases of *D. falcata* leaves, since catechol did not exert any inhibitory effect on either fraction, but actually activated the fraction A enzyme. At the same time, it may be noted that caffeic acid, another dihydric phenol, inhibited both forms of the enzyme. Another point of distinction was the extreme sensitivity of one of the two forms, (B), to the action of phloridzin. Form B phosphorylase resembled muscle phosphorylase[10] and potato phosphorylase[11] in being inhibited by phloridzin. The two phosphorylases of *D. falcata* responded differently towards the phenolics extracted from *Orobanche cernua*. The sensitivity of the two phosphorylases towards phenolics compounds may serve as regulatory mechanism in the cell. Nakamura[11] and Schwimmer[12] also reported inhibition of potato enzyme by phenolics. Schwimmer[12] proposed, without experimental confirmation, that inhibition by phenols may be related to the presence of pyridoxal phosphate in the enzyme.

The phosphorylases present in higher plants do not seem to require AMP for their activity.[6,13] Of the 2 electrophoretically distinct phosphorylases found by Fredrick[14,15] in the blue green alga, *Oscillatoria princeps*, the more mobile phosphorylase (a_2) had a requirement for AMP and Mn^{2+} to effect glucan synthesis. On the contrary, AMP was shown to be inhibitory for one of the forms of phosphorylase of maize endosperm.[16] Fraction A phosphorylase of *D. falcata* resembles muscle phosphorylase *b* in requiring 5'-AMP for full activity and fraction *B* phosphorylase resembles phosphorylase *a* in not requiring AMP. The effect appeared to be specific for AMP, since ATP elicited a different response and the assay system did not have apyrase activity.

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The two forms of *D. falcata* phosphorylases also exhibited certain primer specificities. Fraction A alone could utilize glycogen and fraction B alone used achroic dextrin. The utilization of glycogen as efficiently as soluble starch, preferential utilization of amylopectin, the low efficiency with white dextrin and the non-utilization of achroic dextrin in one case (A), compared to the non-utilization of glycogen and the equal efficiency of white dextrin as that of soluble starch and the use of achroic dextrin, though less efficiently, by the other (B), suggest that while saccharides of higher degree of polymerization are better suited for fraction A, the B form of enzyme could use maltooligosaccharides of low degree of polymerization. Whether one of the enzymes (B) is of special importance in the initiation of polysaccharide biosynthesis in *D. falcata* cannot be stated definitely at present. Recently, Tsai and Nelson[17] speculated that phosphorylase II and III might have a synthetic role in maize endosperm.

Although there were important points of distinction, the two forms of *D. falcata* phosphorylases resembled each other in some properties, such as, inhibition by CN^-, heavy metals, ascorbic acid, bisulfite, dithionite and phenolic products of *D. falcata* leaves, utilization of native starch from a number of sources and non-utilization of maltose as primer. The fraction A and B phosphorylases of *D. falcata* leaves may be considered isozymes on the basis of separation by DEAE-cellulose chromatography,[18] difference in heat stability[19] and kinetic properties.[20] The regulation of the synthesis and the breakdown of starch may be mediated by these isozymes due to their different properties.

EXPERIMENTAL

Starch from different sources, namely, the filaments of *Cuscuta reflexa* Roxb. and the whole growth of *Orobanche cernua* Loefl. infecting respectively *Pithecellobium dulce* (Roxb.) Benth and *Solanum melongena* L. and the seeds of *Oryza sativa* L., *Phaseolus vulgaris* L. and *Zea mays* L. was isolated according to Badenhuizen,[21] Watson[22] and Wolf.[23] Amylopectin from tubers of *Solanum tuberosum* L. was prepared according to Gilbert *et al.*[24] Achroic dextrin fraction was prepared from soluble starch as described by Porter.[2]

The phenolic products from the tissue of a number of angiosperm parasites, namely, *Cuscuta campestris* Yunck, *Cuscuta indecora* Choisy and *C. reflexa* and *O. cernua*, all infecting *Petunia hybrida* X Hort, ex-Vilm. growing on *Mangifera indica* L were prepared as described earlier [25]

Phosphorylase Assay

The enzyme was assayed as described elsewhere,[8] with the difference that the assay volume was reduced to 0·5 ml. After enzyme inactivation (which was by the addition of 0·1 of 10 N H_2SO_4) and charcoal treatment when necessary, the entire solution (no precipitate was formed) was used for color development. Also, the final volume of color solution was reduced to 5 ml from 10 ml, by halving the various reagents. In this way, an overall 4-fold increase in sensitivity of the assay was achieved.

Under the conditions of the enzyme assay, phenolics did not interfere in the determination of activity. Cysteine, glutathione and β-mercaptoethanol also did not interfere, but ascorbic acid, bisulfite and dithionite led to about 20 per cent increase in the phosphorus color.

The specific activity of the enzyme was the activity in units per mg protein.

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Preparation of Crude Extract

All the purification steps were carried out at 2–4°. Grinding of leaf tissues in two stages was carried out as described elsewhere.[8]

Using 100 g leaf tissue, 10% (w/v) dispersion was prepared by grinding in a large sized chilled metal bowl (capacity 1 gal) of Waring blendor with 1000 ml of the 'elimination medium'. The dispersion, without filtration through cloth, was clarified by centrifugation in the cold at 16,000 *g*, for 45 min.

The residual cake was ground with chilled 1000 ml of 'extraction medium', to give 1010 ml of homogenate. One thousand ml of this was centrifuged in the cold at 16,000 *g* for 45 min; the residue this obtained was reground in Waring blendor for 3 min with about 550 ml chilled extraction medium and the suspension centrifuged. The initial extract and the wash fluid were mixed to give 1510 ml of a dark green solution (Fraction I) which served as the starting material. The solution contained about 80 per cent of the phosphorylase activity of the homogenate and had a pH of 7·2.

Ammonium Sulfate Fractionation

$(NH_4)_2SO_4$ was added to 1500 ml of Fraction I to attain 30 per cent saturation (264 g). The pecipitater was discarded after centrifugal sedimentation. An additional 307 g $(NH_4)_2SO_4$ was added (60% saturation) and the suspension centrifuged at 16,000 *g* for 45 min. The precipitate was suspended in 52 ml 0·05 M Tris-HCl buffer. pH 7·5, containing 0·002 M each of freshly neutralized cysteine and EDTA and dialysed for 20 hr against 2·5 l. of 0·005 M Tris-HCl buffer pH 7·2, containing 0·002 M cysteine and EDTA. The resulting suspension was centrifuged at 16,000 *g* for 45 min to give a clear dark green supernatant (Fraction II).

Chromatography on DEAE-cellulose

Thirty ml of $(NH_4)_2SO_4$ fraction (Fraction II) was adsorbed in a DEAE-cellulose column (resin bed volume = 50 ml) which had been equilibrated with 0·005 M tris-HCl buffer pH 7·2. containing 0·002 M each of neutralized cysteine and EDTA. The enzyme was eluted with a stepwise salt gradient consisting of 100 ml portions of the equilibrating buffer system supplemented, respectively, with 0·1, 0·2, 0·3, 0·5 and 1·0 M NaCl. A total of 50 fractions of 10 ml each was collected. The activity in all the fractions was determined separately using achroic dextrin, oyster glycogen and soluble potato starch as primers. Two major peaks of activity were observed using soluble starch as primer; Fraction A eluted with 0·1 M NaCl (major activity in tube numbers 7–10) and Fraction B, with 0·3 M NaCl (major activity in tube numbers 27–30). A minor, but consistent peak, Fraction C, was eluted at 0·2 M NaCl (tube numbers 16 and 17). Using glycogen as primer one major peak of activity was observed which coincided with Fraction A. With achroic dextrin also, one major peak was observed but corresponding to Fraction B. The active tubes of every fraction were mixed and dialyzed against three changes of 2·5 l. each of 0·005 M Tris-HCl buffer, pH 7·2, for 20 hr to remove salt.

Reproducibility of Elution Pattern

The above elution pattern was confirmed by the use of a linear gradient made up of 200 ml of the starting buffer system (0·005 M Tris-HCl buffer, pH 7·2, containing 0·002 M each of cysteine and EDTA) against a gradient of the same volume of buffer system containing 1 M NaCl. Also, on adsorption of the combined active fractions (eluted stepwise) on DEAE-cellulose and rechromatography by stepwise elution, the same pattern of elution was repeated. In this manner, the possibility of formation of column artifacts was eliminated.

Protein Estimation

Protein estimation was carried out as described earlier.[8] For protein profile in the eluted fractions, absorption at 260 and 280 mμ was measured against the respective blanks of 0·005 M Tris-HCl buffer containing NaCl. Calculations were based on the formula used by Kalckar.[26]

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