Phytochemistry, 1974, Vol 13, pp 1359 to 1366 Pergamon Press Printed in England

PURIFICATION AND PROPERTIES OF CELLULASE FROM *PHASEOLUS VULGARIS**

FRANCES T. LEW and LOWELL N. LEWIS†

Department of Plant Sciences, University of California, Riverside CA 92502 U S A

(*Received Revised* 29 *November* 1973)

Key Word Index—*Phaseolus vulgaris*, Leguminosae, kidney bean, carboxy methyl cellulase, purification, isoenzymes, cotyledons, isoelectric focusing

Abstract—Cotyledons of germinating kidney beans contain two forms of a carboxy methyl cellulase which can be separated by ammonium sulfate fractionation and isoelectric focusing The two cellulases are similar in their molecular weight but differ in isoelectric points, pH and temperature optimum, pH and temperature stability and sensitivity to thiol inhibitors and metal ions One cellulase (isoelectric point 4·8) has been purified 100-fold to give a major protein band on acrylamide gel electrophoresis

INTRODUCTION

PLANT cellulases (E.C.3.2.1.4.) probably play an important role in cell development and differentiation, and in the hormonal regulation of these phenomena.[1–8] Recently it was shown that plant cellulase is capable of hydrolyzing plant cellulose and functions as a true cellulase in the plant.[7] Detailed studies on the properties of plant cellulase are not available but are essential to establish the role of this enzyme. Several reports have suggested that plants may possess more than one form of cellulase, but a thorough comparison of the properties of various forms is lacking. Plant cellulases are present in low concentrations and are difficult to obtain in a highly purified form. To our knowledge, the only MW estimations reported for plant cellulases are those by Lewis *et al.*,[6] on the two cellulases isolated from the abscission zone of *Phaseolus vulgaris*.

In this paper, we report the isolation, purification and characterization of two forms of cellulase in the germinating cotyledon of *P. vulgaris*, cv. Red Kidney, which contains a relatively high level of cellulase activity.

* From the Department of Plant Sciences, University of California, Riverside, CA 92502, U S A This work was supported by NSF Grant GB-17850.

† Person from whom reprints should be requested Department of Plant Sciences, University of California, Riverside, CA 92502, U S A

[1] HORTON, R F and OSBORNE, D J (1967) *Nature* **214,** 1086
[2] ABELES, F B and LEATHER, F R (1971) *Planta* **97,** 87
[3] DAKTO, A H and MACLACHLAN, G A. (1968) *Plant Physiol* **43,** 735
[4] SHELDRAKE, A R and MOIR, G F J (1970) *Physiol Planta* **23,** 267
[5] LEWIS, L N and VARNER, J E (1970) *Plant Physiol* **46,** 194
[6] LEWIS, L N, LEW, F T, REID, P D and BARNES, J E (1972) In: *Plant Growth Substances* 1970 (CARR, D J ed), p 234, Springer, Berlin
[7] LEWIS, L N, LINKINS, A E, O'SULLIVAN, S and REID, P D (1974) In *The 8th International Conference on Plant Growth Substances, Tokyo* In Press
[8] LINKINS, A E, LEWIS, L N and PALMER, R L (1973) *Plant Physiol* **52,** 9

RESULTS

Separation of the two molecular forms of cellulases

The distribution of cellulase activities in different enzyme fractions is shown in Table 1. The 10^5 *g* supernatant retained nearly all the cellulase activity present in the 12×10^3 *g* supernatant. About 90% or more of the cellulase was in the supernatant after pH 5·5 treatment, with a 3- to 4-fold gain in specific activity. On ammonium sulfate precipitation, cellulase activity was distributed in three of the four fractions. The 35–45% fraction contained a cellulase (4·5) with a pI of 4·5 on isoelectric focusing (IEF). The cellulase (4·8) in the 55–85% fraction had a pI of 4·8 on IEF. Cellulase activity in the 45–55% fraction was attributable to the cellulases, 4·5 and 4·8. A similar separation of cellulases with ammonium sulfate has been reported in barley and rye [9,10]. IEF treatment of the 10^5 *g* supernatant solution shows both peaks of activity, in the ratio of about 1:3 for cellulase 4·5 and 4·8 respectively.

TABLE 1. THE DISTRIBUTION OF CELLULASE ACTIVITIES IN ENZYME SOLUTIONS AFTER DIFFERENT TREATMENT OF THE CRUDE EXTRACT

Step	Vol (ml)	Total activity (unit/ml/hr)	Conc of protein (mg/ml)	Sp. act. (units/mg protein)	Yield (%)
12×10^3 *g* supernatant	680	33000	48·5	1·0	100
10^5 *g* supernatant pH 5·5	570	32000	21	2·7	97
supernatant $(NH_4)_2SO_4$ 0–35%	590	30000	8·3	6·0	90
25–45%	42	9830	24	9·7	30
45–55%	95	7800	14	5·8	24
55–85%	390	12480	4	8	38

Purification of cellulase 4·8

Cellulase 4·8 was further purified about 100-fold by the steps summarized in Table 2. Cellulase 4·8 did not adsorb on CM-Sephadex equilibrated with 10 mM phosphate buffer, pH 6·0. Nearly all the activity was recovered in the filtrate with about 2-fold purification in this step. Cellulase 4·8 completely adsorbed to DEAE-Sephadex equilibrated with 10 mM Tris–HCl, pH 8·1. The enzyme activity was eluted from the Sephadex gel by a stepwise increase in NaCl concentration from 0·1–0·5 M in this buffer. About 70% of the total adsorbed activity was eluted in 0·2 and 0·3 M NaCl. Specific activity increased 5- to 6-fold over the CM-Sephadex step. The 0·2 and 0·3 M eluates were combined for further purification by two IEF runs in 1% ampholine, pH 4–6. The fractions containing cellulase activities were pooled, sucrose and ampholine were removed and the sample was subjected to electrophoresis on acrylamide disc gel at pH 9·2. Three protein bands were visible after staining with Coomassie Blue. The cellulase activity was detected in a region which coincided closely to the front running band of bromophenol blue ($R_f = 0{\cdot}5$). To obtain highly

[9] PRELL, I. A., AITKEN, R. A. and DICK, J. A. (1954) *J. Inst. Brew.* **60**, 497
[10] MANNERS, D. J. and MARSHALL, J. J. (1973) *Phytochemistry* **12**, 547

purified cellulase 4·8, 400 μg protein from the second IEF run was subjected to preparative electrophoresis. A 5 mm gel segment corresponding to $R_f = 0{\cdot}5$ was removed, and the protein was eluted with 0·5 ml of 0·2 M NaH_2PO_4, pH 4·3. The eluate was subjected to electrophoresis at pH 9·2. One major protein band was detected at $R_f = 0{\cdot}5$.

TABLE 2 PURIFICATION OF THE CELLULASE 4 8

Step	Vol (ml)	Total activity (unit/ml/hr)	Conc of protein (mg/ml)	Sp act (units/mg protein)	Yield (%)
$(NH_4)_2SO_4$ (55–85%)	390	12480	4	8	100
CM-Sephadex	465	12090	2 3	11 3	95
DEAE-Sephadex (0 2 + 0 3 M NaCl)	100	10200	1 7	60	81
Isoelectric focusing (1 ×)	18	6080	1 8	187	48
Isoelectric focusing (2 ×)	10	2400	0 295	814	19

Purification of cellulase 4·5

Cellulase 4·5 was partially purified by the steps summarized in Table 3. There was essentially no increase in specific activity. The overall recovery was also very low, about 1%. Unlike cellulase 4·8, the 4·5 enzyme lost activity gradually at 2° in 20 mM phosphate buffer containing 0·1 M NaCl, pH 7. The enzyme lost considerable activity following lyophilization, dialysis and freezing and thawing. Attempts to reactivate or to stabilize this enzyme by addition of 10^{-3} M dithiothreitol (DTT) with or without 10^{-3} M EDTA were unsuccessful.

TABLE 3 PURIFICATION OF THE CELLULASE 4 5

Step	Vol (ml)	Total activity (unit/ml/hr)	Conc of protein (mg/ml)	Sp act (units/mg protein)	Yield (%)
$(NH_4)_2SO_4$ 35–45%	42	9828	24	9 7	100
CM-Sephadex	85	5908	6	11 5	70
$(NH_4)_2SO_4$sat 35–45% (2 ×)	14 5	774	17	3 1	8
Bio-Gel p-150	22	275	1 22	10 1	3
Isoelectric focusing	17 5	114	0 375	17	1 1

The enzyme fraction from isoelectric focusing was subjected to electrophoresis on a disc gel at pH 9·2. One major protein band ($R_f = 0{\cdot}49$) and a few minor bands were observed in the gel. A small but definite amount of cellulase activity was also recovered in the region corresponding to this major protein band. A large amount of the cellulase activity was found at the origin of the gel, suggesting that cellulase 4·5 did not migrate into the gel.

Properties of the partially purified cellulases 4·5 and 4·8 optimum pH

The enzymes used in the present study were purified through the ion exchange Sephadex treatments shown in Table 2 or 3. The two forms of cellulase differ in their pH requirements for maximal enzymic activities. The optimal pH for cellulase 4·5 was 5·7–6·2, while that for the cellulase 4·8 was 4·8–5·6. Others have found pH optimal for plant cellulases.[11,12]

pH Stability

In contrast to its relatively unstable nature, cellulase 4·5 had a broader pH stability curve than cellulase 4·8. The former was relatively stable in the pH range from 6 to 9, while the latter is stable only from 6·5 to 8. Cellulase 4·5 was most stable at pH 8 and half activity was lost at pH 5·5 and 9·5, while cellulase 4·8 was not stable at pH 7 and half activity was lost at pH 6 and 8·8.

Optimal temperatures

The optimal temperature for hydrolysis of CMC for cellulase 4·8 was from 35 to 43°. The optimal temperature for cellulase 4·5 was about 45–60°.

Temperature stability

Enzyme activity decreased for both cellulases at temperatures above 30° when incubated at various temperatures for 30 min in the absence of substrate, then cooled and assayed at 38–40°. Cellulase 4·8 loses about half of its initial activity after 30 min at 36°. On the contrary, cellulase 4·5 retains at least 75% of its original activity under the same condition. These data also demonstrate the protective effect of the CMC substrate on the stability of cellulase at high temperatures.

TABLE 4. EFFECT OF THIOL INHIBITORS ON THE CELLULOSE ACTIVITY AS % OF CONTROL

Conc (Molar)	Cellulase 4·5		Cellulase 4·8	
	NEM	*p*-CMB	NEM	*p*-CMB
10^{-6}	100	90	100	100
10^{-5}	90	65	100	90
10^{-4}	60	15	100	55
10^{-3}	40	--	98	
10^{-2}	30	--	90	

The reaction mixture of cellulase 4·5 contained about 10 units of purified enzyme, 0·2 M citric-phosphate buffer pH 5·9, CMC and NEM at 10^{-6}–10^{-2} M. The reaction mixture for cellulase 4·8 was the same except the pH was 5. Reaction mixtures without the thiol inhibitor were included as controls. Enzyme activity was measured after incubations at 39–40° for 30 min.

Effect of thiol reagents

The thiol inhibitors *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate (*p*-CMB) have previously been reported to inhibit some fungal, microbial and plant cellulases.[6,13] NEM had essentially no effect on cellulase 4·8 but it caused 10, 40, 60 and 70% inhibition

[11] SANDEGREN, E. and ENEBO, L. (1952) *J. Inst. Brew.* **58**, 198.
[12] MOFFA, D. J. and LUCHSINGER, W. W. (1970) *Cereal Chem.* **47**, 54.
[13] MANDELS, M. and REESE, E. T. (1963) In *Advances in Enzymatic Hydrolysis of Cellulose and Related Materials*. (REESE, E. T., ed.) pp. 115–158. Macmillan, New York.

of the 4·5 enzyme when included in the assay medium at a concentration of 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M respectively (Table 4).

p-CMB inhibited both enzymes but to a different degree. At 10^{-4} M, the highest concentration used, *p*-CMB decreased the cellulase 4·5 activity by 90% but cellulase 4·8 was reduced only 50% (Table 4).

When DTT was included with the thiol reagents at concentrations from 10^{-5} to 10^{-2} M, it had no effect on the activities of either form of the enzyme. But when the cellulase 4 5 was pre-incubated with 10^{-3} M DTT for 72 hr, then assayed in the presence of 10^{-4} M *p*-CMB, the enzyme activity was decreased by only 50% instead of 90% as in the previous case.

Effect of metal ions and cellobiose

Both enzymes were strongly inhibited by Ag^{+} and Hg^{2+} ions at concentrations of 10^{-4} and 10^{-3} M (Fig. 1). At 10^{-6} M, these heavy metal ions caused a 50% inhibition of the cellulase 4·8 while their effect on the cellulase 4·5 was nil.

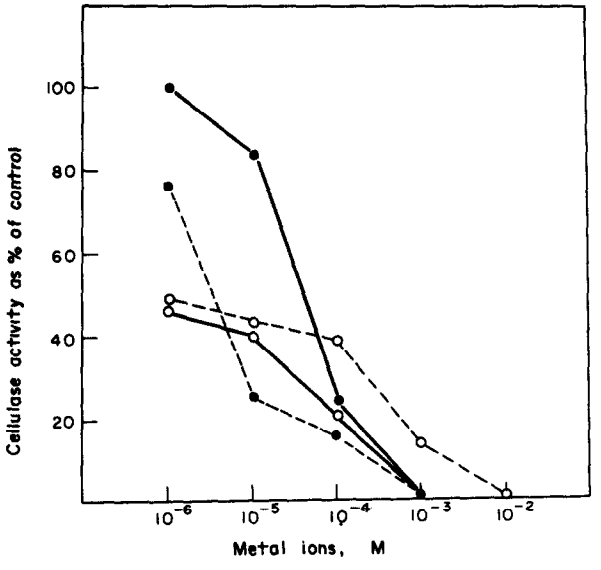

FIG 1 EFFECT OF Ag^{+} AND Hg^{2+} ON THE ACTIVITIES OF CELLULASES 4·5 AND 4 8.
The reaction mixture of the cellulase 4 5 (solid symbols) contained about 10 units of enzyme in 0 2 M citric-phosphate, pH 5 9, CMC and Ag^{+} (solid line) or Hg^{2+} (dashed line) ions at 10^{-6} to 10^{-2} M The reaction mixture of cellulase 4 8 (open symbols) was the same except the pH was 5 Reaction mixtures without Ag^{+} or Hg^{2+} ions were included as controls Enzyme activity was measured after incubations at 39–40° for 30 min.

Of the four other divalent ions tested (Table 5) only Cu^{2+} at 10^{-3} M showed appreciable inhibition of the cellulase 4·8. The other metal ions showed no effect. All four divalent metal ions, however, caused appreciable inhibition of the cellulase 4·5 activity. The Cu^{2+} and Zn^{2+} ions reduced this enzyme activity by 84 and 73% respectively, while Mg^{2+} and Mn^{2+} diminished its activity to a lesser extent. Such effects of metal ions should be considered in establishing *in vitro* conditions for cellulase enzyme studies.

Cellobiose has been reported as an end-product inhibitor in some fungal cellulases.[13] The effect of this sugar on plant cellulase is not well documented. Preliminary experiments indicated that cellobiose caused a 40% inhibition of cellulases 4·5 and 4·8 when this sugar was included in the assay medium at a concentration of 1% or more. This concentration is greater than can be obtained from our assay mixture and so did not interfere with our enzyme assay.

TABLE 5 EFFECT OF DIVALENT METAL IONS ON THE ACTIVITIES OF CELLULASES 4·5 AND 4·8

Metal ions (5×10^{-3} M)	Inhibition % compared to control	
	4·5	4·8
0	0	0
Zn^{2+}	73	0
Mg^{2+}	10	0
Mn^{2+}	20	0
Cu^{2+}	84	28

Reaction mixtures for cellulase 4·5 assay contained 8 units of the purified enzyme in 0·1 ml, 1 ml 1·21% CMC, 0·4 ml 0·2 M citric-phosphate buffer, pH 5·9 and 5×10^{-3} M of the metal ions. The reaction mixture for cellulase 4·8 were as above, except that the buffer was 0·4 ml of 0·2 M citric-phosphate pH 5·0. Incubated at 39–40° for 1 hr.

Molecular weight of cellulases 4·5 *and* 4·8

A semi-log plot of MW against elution volume using three marked enzymes showed that the cellulases 4·5 and 4·8 had a MW of about 7×10^4.

DISCUSSION

Cotyledonary cellulase exists in two forms designated cellulases 4·5 and 4·8 with isoelectric points of 4·5 and 4·8 respectively. These two forms are similar in molecular weight and in catalytic action on carboxymethyl cellulose; in other properties tested, however, they are different. In addition to isoelectric points, the difference is manifested in pH and temperature optimum, pH and temperature stabilities, solubility in ammonium sulfate, sensitivity toward thiol group inhibitors and sensitivity to metal ions such as Ag^+, Hg^{2+}, Cu^{2+} and Zn^{2+}.

Although the cellulase 4·5 is stable in broader ranges of pH and temperature than the 4·8 enzyme, it is much less stable than the latter during storage, dialysis, CM-Sephadex treatment, gel filtration and isoelectric focusing.

Cellulase 4·5 is also more sensitive to NEM and *p*-CMB. Both forms of the enzyme are more sensitive to *p*-CMB than NEM. Since *p*-CMB is more effective on sulfhydryl groups in the interior of the molecule than NEM,[14] this suggests that enzymic activity is dependent upon internal sulfhydryl groups in both forms of cotyledon cellulase.

Cellulase is not known to require any metal ion for its enzymic activity, but metal ions, especially those of heavy metals, are inhibitory to many of the fungal cellulases.[15,16] The heavy metal ions, Ag^+ and Hg^{2+} at 10^{-4} M inhibit both forms of cotyledonary cellulases by 80%. Cellulase 4·5 was the more sensitive form to the divalent metals, Zn^{2+} and Cu^{2+}.

[14] FRAENKEL-CONRAT, H (1957) In *Methods in Enzymology* (COLOWICK, S P and KAPLAN N O, eds) Vol IV, p 268, Academic Press, New York

[15] IWASAKI, T IKEDA R, HAYASHI K and FUNATSU, M (1965) *J Biochemistry* **57**, 478

[16] MANDELS M and REESE E T (1966) *Ann Rev Phytopathol* **3**, 85

EXPERIMENTAL

Assay Cellulase was assayed by measuring the reduction in viscosity of a 1·21% solution of CM-cellulose (CMC) type 7HF as described earlier [5] The assay mixture contained 2 parts of CMC soln in 20 mM sodium phosphate buffer, pH 6·1 and 1 part of the enzyme soln The reaction mixture was usually incubated in a 38–39° bath for 30–60 min Viscosity was measured after equilibration to room temp Viscosity data were converted to relative units of activity as described in Almin *et al* [17] Assays were replicated 2 or 3×

Germination. Red kidney beans were surface sterilized for 15 min with 1% (v/v) Clorox (sodium hypochlorite) in tap H_2O The seeds were soaked in H_2O for 30 min and placed on H_2O saturated filter paper in a pre-sterilized plastic box The beans were allowed to germinate in the dark at 23–24° for 2–3 days before harvesting

Preparation of enzyme Step 1 Extraction The cotyledons were ground in a Waring blender with 20 mM phosphate buffer, pH 6·1. The homogenate was pressed through several layers of nylon cloth and the residue was washed 3× with the same buffer The amount of buffer used for initial extraction was usually 2× that of the cotyledon fresh weight and, for each washing, was half that used for initial extraction The initial extract plus the washings were combined and centrifuged at $12 \times 10^3 g$ for 20 min The supernatant solution was subsequently centrifuged at $10^5 g$ for 1 hr

Step 2 pH 5·5 precipitation Citric acid was stirred slowly into the $10^5 g$ supernatant soln until the desired pH was obtained After standing at this pH for 30 min, the ppt was collected by centrifugation at $12 \times 10^3 g$ for 20 min

Step 3 Ammonium sulfate fractionation The supernatant soln obtained for pH 5·5 precipitation was fractionated by $(NH_4)_2 SO_4$ When the salt had dissolved, the soln was allowed to stand undisturbed for about 2 hr, and the ppt was collected by centrifugation at $12 \times 10^3 g$ for 20 min. Four protein fractions were made with $(NH_4)_2 SO_4$, i e 0–35%, 35–45%, 45–55% and 55–85% Each ppt was dissolved in 20 mM phosphate, pH 7, containing 0·1 M NaCl and dialyzed against the same buffer

Step 4 CM-Sephadex treatment CM-Sephadex, C-50, was equilibrated with 10 mM phosphate, pH 6·0 The enzyme soln containing cellulase activity was dialyzed against the same buffer and then added to the gel slurry After 30 min mixing, the gel was filtered and washed with a small amount of the same buffer The filtrate plus the washing was analyzed for cellulase activity

Step 5 DEAE-Sephadex treatment DEAE-Sephadex A-50 was equilibrated with 10 mM Tris–HCl, pH 8·1 The enzyme soln containing cellulase activity was dialyzed against the same buffer and added to the gel slurry After 30 min mixing, the gel slurry was filtered and washed with a small amount of the same buffer Cellulase activity was eluted by increasing the concentration of NaCl from 0·1 to 0·5 M in 0·01 M Tris–HCl buffer, pH 8·1

Step 6 Isoelectric focusing This was carried out according to the procedure described by Haglund [18] Samples were first dialyzed against 1% glycine and applied to 1% ampholine soln, pH 3–10 or pH 4–6 in a sucrose density gradient For pH 3–10, samples were focused at 300 V, and for pH 4–6, at 700 V for 34–40 hr In some experiments, samples were focused at the above voltages for only 24 hr No difference in results was observed in these two experimental conditions The IEF column was drained at a flow rate of 1 ml/1 min and 2 ml fractions were collected The pH of each fraction was measured at 4–8° and neutralized to pH 5·8–6 for cellulase assay

Physical methods The MWs of the isoenzymes were estimated by gel filtration,[19,20] through a calibrated column (2·1 × 73 cm) of Bio-gel P-150, which was equilibrated and developed with 20 mM Tris–HCl, pH 7, containing 0·1 M NaCl The enzyme preparation (3 ml) was applied to the column Fractions (2 ml) of the eluate were collected at a flow rate of 11 ml/hr Marker enzymes used for calibration were: liver alcohol dehydrogenase (MW = 84000), bovine hemoglobin (MW = 67000) and heart cytochrome C (MW = 12500) The enzyme activity of the dehydrogenase was measured spectrophotometrically by following an increase in absorption of NADH at 340 nm using 95% EtOH as substrate Hemoglobin and cytochrome C were followed by their characteristic absorption at 410 nm

Disc gel electrophoresis was performed according to a modified method of Davis[21] in which 50 mM Tris–glycine, pH 9·2, was used as the buffer Acrylamide (5%) plus bisacrylamide gels of (7 × 70 mm) were polymerized chemically by ammonium persulfate and aged for 1 hr before use Prior to addition of protein solns, gels were pre-run at 200 V for 50–60 min Sample solns in 10 to 50 μl containing 20% sucrose were layered directly over the gel surface 50 V was used initially giving a current of 1 mA/gel After 30 min this was increased to 200 V (4 mA/gel) for another 90 min Bromophenol blue soln (0·01%) was used as a tracking dye Protein bands in gel were detected by staining with 0·05% Coomassie Blue in 12·5% TCA as described by Chrambach *et al* [22] For recovery and assay of cellulase activity after electrophoresis, the gel was sliced into 1 mm slices from the cathode end Two gel slices were combined and transferred to a test tube containing 0·5 ml of 0·2 M NaH_2PO_4, pH 4·3

[17] ALMIN, K E AND ERICKSSON, K E (1967) *Biochem Biophys Acta*
[18] HAGLUND, H (1967) *Science Tools* **14,** 17
[19] ACKERS, G K (1964) *Biochemistry* **3,** 723
[20] TING, I P (1968) *Arch Biochem Biophys* **126,** 1
[21] DAVIS, B J (1964) *Ann NY Acad Sci* **121,** 404
[22] CHRAMBACH, A R, REISFELD, A, WYCKOFF, M and ZACCARI, J (1967) *Anal Biochem* **20,** 150

The gel slices were left in this buffer for 18–34 hr then 1 ml CMC was introduced into the soln. Reduction in viscosity was measured after incubation at room temp. Protein solns were concentrated either by lyophilization or by dialysis against powdered polyethylene glycol (Carbowax).

The amount of protein in soln was measured either by biuret or spectrophotometric methods.[23] Desalting of sucrose, and ampholine was achieved by dialysis and by gel filtration on a Bio-gel P-2 or P-10 column. All operations were carried out at 2° unless otherwise mentioned in text.

Acknowledgements—The authors wish to thank Drs. George McNew and Alva App for the use of their facilities at Boyce Thompson Institute for Plant Research, in performing some of the experiments reported in this paper. Carboxymethyl cellulose type 7HF was a gift from Hercules Powder Co., Wilmington, Delaware.

[23] Layne, E. (1957) In *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. III, pp. 450–454. Academic Press, New York.