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CHLOROPHYLLASE OF SUGAR-BEET LEAVES

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Abstract—The chlorophyllase of sugar-beet leaves was characterized and partly purified by gel-filtration and ion-exchange chromatography on various types of Sephadex. A rapid method was developed for determining the chlorophyllase activity of fractions from columns. Indications of more than one form of the enzyme may be explained by its tendency to associate or aggregate with itself or with other substances. Association is greater in 0·02 M citrate than in citrate containing 0·5 M NaCl. The enzyme is strongly adsorbed to negatively charged materials, which may explain its improved extractibility when the sodium chloride concentration of the medium is increased. Measurements of some properties of the enzyme are affected by insolubility of the substrate.

INTRODUCTION

THE ENZYME chlorophyllase (EC 3.1.1.14) catalyses *in vitro* the hydrolysis of the ester linkage between phytol and the C-7 propionic acid group of chlorophylls *a* and *b*, to form the corresponding chlorophyllides. In aqueous alcoholic solvents transesterification also occurs. Reversal of the hydrolytic reaction in the presence of excess phytol was reported by Willstätter and Stoll[1] and by Shimizu and Tamaki.[2] It is not yet clear whether *in vivo* the enzyme catalyses the esterifying or the hydrolytic reaction, or both.

Soluble chlorophyllase preparations have been made from several plant species.[3–10] Sugar-beet leaves are a rich source of the enzyme and we have extended earlier work in an attempt to further characterize and purify the chlorophyllase of this species by various chromatographic procedures.

RESULTS

Characterization of Crude Extracts from Acetone Powders

Crude extracts from acetone powders were concentrated to various extents by ultrafiltration and analysed by gel-filtration on Sephadex G-100. The extraction medium and developing buffer was 0·02 M sodium citrate. Chlorophyllase activity was recovered in many fractions (Fig. 1). This probably means that the activity is associated with high-molecular

[1] R. WILLSTÄTTER and A. STOLL, *Investigations on Chlorophyll* (translated by F. M. SCHERTZ and A. R. MERZ), Science Press, Lancaster, Pa. (1928).
[2] S. SHIMIZU and E. TAMAKI, *Archs Biochem. Biophys.* **102**, 152 (1963).
[3] M. HOLDEN, *Biochem. J.* **78**, 359 (1961).
[4] M. HOLDEN, *Photochem. Photobiol.* **2**, 175 (1963).
[5] M. HOLDEN, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN) p. 461. Academic Press, London and New York (1965).
[6] A. KLEIN and W. VISHNIAC, *J. biol. Chem.* **236**, 2544 (1961).
[7] S. SHIMIZU and E. TAMAKI, *Bot. Mag., Tokyo* **75**, 480 (1962).
[8] P. BÖGER, *Phytochem.* **4**, 435 (1965).
[9] N. OGURA and A. TAKAMIYA, *Bot. Mag., Tokyo* **79**, 588 (1967).
[10] A. K. STOBART and D. R. THOMAS, *Phytochem.* **7**, 1963 (1968).

weight material in the fast-moving fractions and low-molecular weight material in the slower-moving fractions. There is, however, the possibility that the slow-moving fractions could have been absorbed slightly by the Sephadex, although experiments with purified enzyme make this unlikely. The relative amounts of the faster and slower fractions (F and S in Fig. 1) varied in different preparations; the amount of F was usually smaller with lower concentrations of total protein. When the combined lower-molecular weight fractions (S) were concentrated by ultrafiltration and re-run on G-100 there was little of the fast-moving chlorophyllase and there seemed to be less heterogeneity of molecular weight in the slow-moving

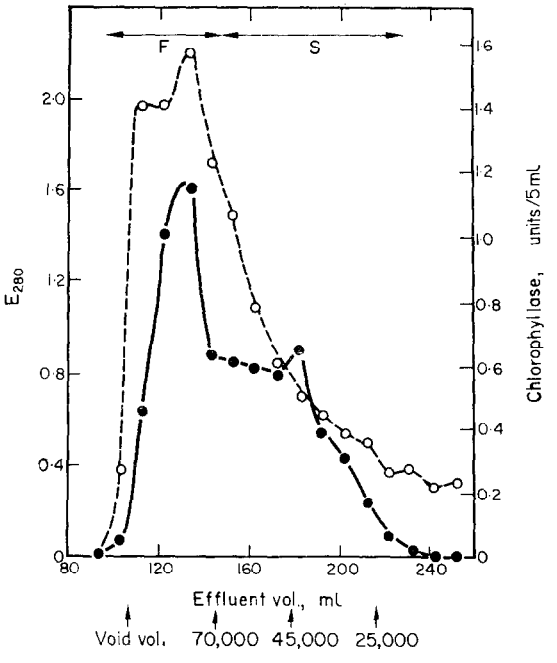

FIG. 1. GEL-FILTRATION ON SEPHADEX G-100 OF THE TOTAL ULTRAFILTERED EXTRACT FROM 20 g OF ACETONE POWDER.

●, Chlorophyllase activity; o, E_{280} (corrected value as described in the text). Approximate molecular weights are indicated by arrows. 6 ml of solution containing about 300 mg of protein were applied, and the column was developed with 0·02 M sodium citrate. NaCl was not present in the extraction medium nor in the developing buffer. F are the fast-moving high-molecular weight fractions; S are the slow-moving low-molecular weight fractions.

peak. When the combined higher-molecular fractions (F) were ultrafiltered and re-run on G-100, some of the enzyme emerged in the lower-molecular weight region. Low-molecular weight chlorophyllase was also obtained when the F fraction was precipitated with 65% acetone at 0° and run on G-100.

The results suggest a partly reversible association of chlorophyllase with itself or with other proteins. The less pronounced association in the re-run fractions might be due to ageing or to smaller concentrations of total protein or specific proteins.

To study the effect of concentrating by ultrafiltration, the gel-filtration pattern for 5 ml of a crude extract was compared with that for 63 ml concentrated to 5 ml (Fig. 2). The more concentrated solution gave relatively less low-molecular weight chlorophyllase, and more

intermediate and higher-molecular weight material. This suggested that association interactions were indeed more marked at greater protein concentrations. The amount of protein in the experiment shown in Fig. 1 was even larger (about 300 mg) than used in curve 2 of Fig. 2, which probably explains the more complex gel-filtration pattern. The relative viscosity of the more concentrated solution in Fig. 2 was only 1·2 at 4°, so that viscosity effects are not likely to be the cause of the changed pattern. When a portion of the ultrafiltered extract was rediluted to the same protein concentration as the original extract, and 5 ml were run on G-100, the gel-filtration pattern then resembled that of the original extract (curve 1 in Fig. 2). However, in another experiment, an enzyme solution held for longer in the concentrated state before redilution gave an increased proportion of high-molecular weight material. It therefore seems that although association was partly reversible, more permanently aggregated material could be formed in concentrated solutions.

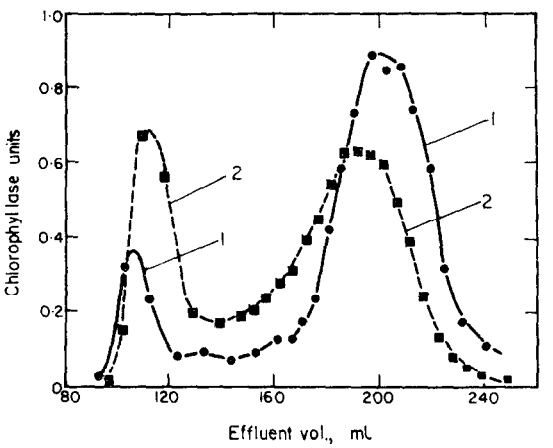

FIG. 2. EFFECT OF ULTRAFILTRATION ON G-100 GEL-FILTRATION PATTERN FOR CHLOROPHYLLASE.
Curve 1 (●, units/60 ml) was obtained with 5 ml of crude extract containing about 10 mg of protein; Curve 2 (■, units/7 ml) was obtained with 63 ml of the same extract concentrated to 5 ml by ultrafiltration (about 120 mg of protein). The extracting medium and developing buffer was 0·02 M sodium citrate, without NaCl. The enzyme activities in the two curves were chosen on an arbitrary basis, for purposes of graphical representation.

Besides formation of chlorophyllides during the assay, there was some indication of chlorophyll bleaching by the high-molecular weight fractions. This was more marked with the related variety, spinach beet. Enzymic bleaching of chlorophyll was observed previously in barley and *Heracleum* leaves suspended in aqueous acetone.[11]

An improved gel-filtration pattern, with a narrower low-molecular weight peak and less high-molecular weight enzyme than in curve 2 of Fig. 2, was obtained when a concentrated extract was run on G-100 in the presence of 0·5 M sodium chloride, which presumably lessened the tendency to associate. Unless otherwise noted, 0·02 M sodium citrate containing 0·5 M sodium chloride was therefore used for gel-filtration on G-100. The high ionic strength of this solution had the added advantage of minimizing interactions between chlorophyllase and the negatively charged groups that are present in small amounts even on "neutral" Sephadex. This could be important because some chlorophyllase was lost when new G-100 columns were run for the first time with 0·02 M sodium citrate alone.

[11] M. F. BACON and M. HOLDEN, *Phytochem.* 6, 193 (1967).

The yield of chlorophyllase was increased by 60 per cent when 0·4 M NaCl was included in the medium used to extract the enzyme from the acetone powder; total protein in the region of the low-molecular weight peak was increased by only 33 per cent. Gel-filtration showed that 17 per cent of high-molecular weight enzyme was present in a sodium chloride-free extract, but only 10 per cent in extracts containing 0·5 M and 0·25 M NaCl.

Characterization of Extracts made from Fresh Leaves

When fresh sugar-beet leaves were ground in 0·02 M sodium citrate, with or without 0·4 NaCl, the centrifuged extract contained an amount of chlorophyllase similar to that obtained when an equivalent weight of acetone powder was extracted with the same solutions. The gel-filtration patterns, including the position of the low-molecular weight chlorophyllase peak, were also similar to those of extracts from acetone powders. Solvent extraction of sugar-beet leaves is therefore not essential for soluble enzyme to be liberated. Extracts from acetone powders were, however, preferred for chlorophyllase purification because the specific activity was much greater than in extracts from fresh leaves, and smaller volumes of liquid could be used for a given amount of enzyme. Acetone powders were also convenient because they could be prepared during the growing season and stored at 0° without loss of activity.

Purification of Chlorophyllase

(a) *Initial concentration and purification on carboxymethyl–Sephadex.* Adsorption on CM–Sephadex proved a convenient method of concentrating chlorophyllase in crude extracts. Much contaminating protein was removed so that when the enzyme was eluted it could be further concentrated by ultra-filtration with fewer complications from association interactions; most of the brown colour was also removed. Analysis by gel-filtration on G-100 showed that 70 per cent of the low-molecular weight but less than 20 per cent of the high-molecular weight chlorophyllase was adsorbed by the ion-exchanger. More than 70 per cent of the total protein passed through. A further 5–10 per cent of the low-molecular weight

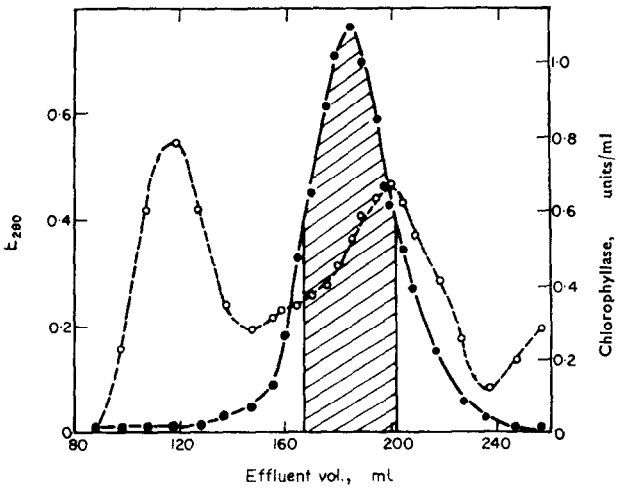

FIG. 3. GEL-FILTRATION ON G-100 OF THE MATERIAL RETAINED BY CM–SEPHADEX.

●, Chlorophyllase activity; o, E_{280}. The 0·02 M sodium citrate–0·5 M NaCl eluate from initial purification and concentration on CM–Sephadex of the extract from 50 g of acetone powder was concentrated by ultrafiltration and run on G-100 with the same buffer. The solution (17 ml) contained about 75 mg of protein.

enzyme was retained either when the extract was passed a second time through CM–Sephadex, or the amount of ion-exchanger was doubled. The rest of this form of the enzyme was not retained when the pH of the initial extract was adjusted to 5·2 and diluted to even lower ionic strength before being run. The low-molecular weight chlorophyllase not retained on CM–Sephadex might represent a distinct form of the enzyme rather than the same form carried through the column by association with other substances.

(b) *Gel-filtration on Sephadex G-100.* The material eluted from CM–Sephadex was concentrated a further eightfold by ultrafiltration and passed through G-100 (Fig. 3). Little or no high-molecular weight chlorophyllase was present. The fractions in the hatched area in the figure were pooled and stored at 4° for short periods or at −18° when several preparations were being made. At this stage in the purification little activity was lost on storage for several months at −18°. However, gel-filtration showed that some high- and intermediate-molecular weight forms were formed; in one preparation there was 35 per cent conversion in 4 months.

(c) *Further purification on CM–Sephadex.* After gel-filtration on G-100 the preparation was again absorbed on CM–Sephadex and this time was eluted with a linear gradient of sodium chloride. The result is shown in Fig. 4. Although the chlorophyllase separated from much other protein, it was clearly still impure because the specific activity at various positions on the enzyme peak was not constant. Enzyme and contaminating protein were less well separated with adsorption and elution at pH 7. Gel-filtration on G-100 showed that the material was of low molecular weight.

Table 1 summarizes the yields and extent of purification obtained by the above means.

TABLE 1. PARTIAL PURIFICATION OF CHLOROPHYLLASE

	Protein (mg)	Chlorophyllase (units)	Specific activity (units/mg protein)	Yield (%)
Citrate–NaCl extract	1000	91·0	0·091	100
After first treatment with CM–Sephadex	89	42·7	0·48	47
After G-100 gel-filtration	14	22·4	1·60	25
After gradient elution from CM–Sephadex (hatched area of Fig. 4)	2·6	10·6	4·08	12

The starting material was 50 g of solvent extracted sugar-beet acetone powder, containing 4·7% N. Chlorophyllase rejected as headings and tailings from the material collected at each stage is not included in the recoveries shown.

Chromatography on QAE- and DEAE-Sephadex

In attempts to further purify the enzyme, Sephadex anion-exchangers were tested. Chlorophyllase was not readily retained and a dilute buffer solution of pH 8·9 was therefore used to increase adsorption. With both the anion-exchangers there were two main chlorophyllase peaks. About 40% of the enzyme was eluted without adding salt and most of the remainder needed less than 0·1 M NaCl. Figure 5 shows a typical result for DEAE-Sephadex using a

preparation partly purified on CM–Sephadex followed by G-100. Much protein precipitated during the preliminary ultrafiltration and dialysis against the same dilute buffer that was used for developing the column. The first peak was not caused by overloading; the percentage of activity in this peak was not increased when 4 mg was applied instead of 1 mg.

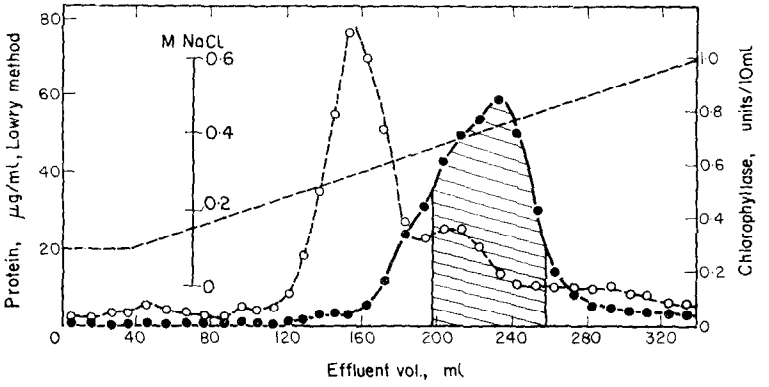

FIG. 4. ION-EXCHANGE CHROMATOGRAPHY ON CM–SEPHADEX OF POST-G-100 MATERIAL.

●, Chlorophyllase activity; o, protein concentration; ---, molarity of NaCl entering the column. The developing buffer was 0·02 M citrate with increasing amounts of NaCl (pH 5·8–5·4). The column loading (6 mg protein in 6 ml solution) corresponds to the material purified from 20 g of acetone powder.

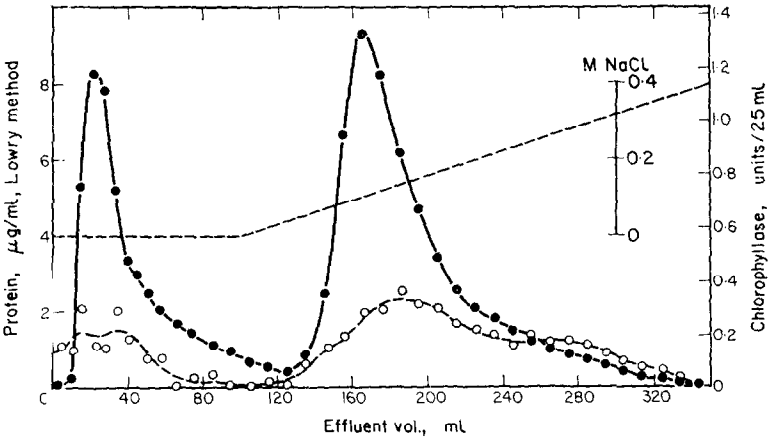

FIG. 5. ION-EXCHANGE ON DEAE–SEPHADEX AT pH 8·9 OF THE MATERIAL IN THE HATCHED AREA OF FIG. 3.

The column was developed initially with 0·025 M tris–0·005 M HCl, then with a linear gradient of NaCl in the same buffer. ●, Chlorophyllase activity; o, protein concentration; ---, molarity of NaCl entering the column.

The presence of two peaks might mean that two different low-molecular forms of the enzyme were present. Alternatively, aggregated or associated material might have been formed either during the preliminary dialysis or on the anion-exchanger because of the dilute buffer used. Experiments with gel-filtration on G-100 were complicated by poor recoveries and failed to resolve which of these possibilities was the correct one. Properties of the enzyme were studied without purification on anion-exchangers.

Properties of Chlorophyllase

Molecular weight by gel-filtration. When run on a column calibrated with proteins of known molecular weight the elution volume of the low-molecular peak was between 190 and 203 ml. This corresponds to a molecular weight of 30,000–38,000 assuming that chlorophyllase behaves as a typical globular protein. When the protein concentration was large the elution volume was up to 5 per cent less than with small concentrations. The rather wide range of values with low concentrations, obtained with fractions of different purity, could be due to more than one low-molecular weight form of the enzyme.

General properties. The enzyme was strongly retained on cation-exchange materials but not on anion-exchangers, and so might be expected to be basic. Electrophoresis on cellulose acetate membranes in 0·01 M citrate at various stages of purification suggested, on the contrary, that it was neutral or even slightly acidic. Recoveries of activity from the membranes were, however, not good and with the purer preparations the enzyme remained at the application point over the pH range 5·4–7·8. It may be adsorbed by the membrane and the electrophoresis results would be further invalidated if the charge on the enzyme is modified by association with the buffer or with other proteins.

The material obtained after the second run on CM–Sephadex was used for studying further the properties of the enzyme. The broad peak shown in Fig. 4 may have been due to multiple forms of chlorophyllase any of which could be artifacts. Thus the right-hand side of the peak, although containing the purer enzyme, was not necessarily the most representative of chlorophyllase in the leaf. We therefore used the fraction contained in the hatched area of Fig. 4 even though it included much of the contaminating protein peak at 210 ml.

Stability. The final preparations were reasonably stable between pH 5·6 and 8·9, especially when kept in stoppered flasks with only a little air space. Dilute solutions were less stable than more concentrated ones; at a protein concentration of 50 μg/ml about 25% of the activity was lost in 30 days at 4°, whereas at 2 μg/ml the same amount was lost in 1 day. At the latter concentration the activity was not stabilized by 0·1% cysteine, 0·01 M mercaptoethanol, 20% glycerol, 0·025 M EDTA or by storage at −18°. Glycerol 50% gave partial protection; with bovine serum albumin, 0·75 mg/ml, there was no loss of activity during 5 days at 4°. An unexplained immediate loss of activity sometimes occurred on diluting.

When heated for 5 min in 0·02 M citrate – 0·18 M NaCl (pH 6·3) at 30 μg protein/ml all activity was lost between 60 and 70°. In aqueous acetone solutions containing sodium citrate no activity was lost in 30 min at 21° at acetone concentrations between 10 and 55%; one-third of the activity was lost in 70% acetone.

Effect of acetone concentration on activity. The acetone concentration for optimum activity depended on the test conditions; these included alteration of the amount and concentration of the substrate when added, and variation in the concentration of salts and acetone before adding the substrate. It was not always reproducible even under fixed assay conditions. In most tests the optimum was at about 45% of acetone but in some it was only 25%. These observations might be explained by differences in the state of aggregation and accessibility of the chlorophyll, which is in suspension rather than in true solution.

pH and temperature optima. The variation of activity with pH and temperature was measured previously in high concentrations of acetone.[3] Because heat stability of the enzyme was affected by 40% acetone, it seemed more meaningful to measure pH and temperature optima at a low concentration of organic solvent. With 2 per cent of acetone in citrate buffer the activity was only 30% or less that measured in 40% acetone.

Klein and Vishniac[6] measured chlorophyllase activity at 30° using 40 μg pheophytin *a* or chlorophyll *a* as substrate in 1 ml of phosphate buffer containing 2% diethyl ether and 0·2% of the non-ionic detergent Triton X-100. We found that conversion of purified pheophytin *a* was no better under their conditions than with citrate buffer containing 2% acetone but no Triton. However, the equally poor conversion of purified chlorophyll *a* in 2% acetone–citrate was improved by adding the detergent, although at 21° it was still only about one-half of that obtained with 40% acetone–citrate.

Using Klein and Vishniac's conditions with chlorophyll *a* as substrate, the temperature for maximum activity was about 40° at pH 7·6. The reaction time was 30 min and the extent of conversion was measured by our chromatographic method. The optimum pH at 36° was 7·1 (±0·5). No appreciable amounts of magnesium-free derivatives were formed in these tests.

Specificity. "Changed" chlorophylls, possibly oxidized at the C-10 position,[11,12] were tested earlier as substrates for chlorophyllase. Left for 15 hr in 50% aqueous acetone with an acetone powder of *Lunaria biennis* leaves, "changed" chlorophyll *a*-2 seemed not to be hydrolysed whereas "changed" chlorophyll *a*-1 was largely converted into a pigment with a lower R_f than chlorophyllide *a*.[11] In the present work "changed" chlorophylls *a*-1, *a*-2 and *a*-3 were prepared by alteration of chlorophyll *a* on silica gel,[12] and purified by TLC on cellulose. They were then tested with purified sugar-beet chlorophyllase in 40% acetone. With amounts of enzyme giving almost complete hydrolysis of chlorophyll *a* in 30 min, there was perhaps 2 per cent conversion of "changed" chlorophyll *a*-1 and no obvious alteration of *a*-2 and *a*-3. It seems therefore that the "changed" chlorophyll *a* compounds *a*-1, *a*-2 and *a*-3 are either not substrates for chlorophyllase or are hydrolysed only very slowly.

DISCUSSION

The chlorophyllase of sugar beet, and of other species, requires a high concentration of organic solvent for optimum activity *in vitro*. This may be related to the accessibility of the substrate rather than being an intrinsic property of the enzyme. The insolubility of chlorophyll *a* and other potential substrates in aqueous media, or even in 40% acetone, can be expected to cause difficulty in measuring some of the properties of the enzyme. With test solutions containing chlorophyll *a* and only 2% of organic solvent, the activity was improved by adding the detergent Triton X-100, as used by Klein and Vishniac.[6] Activity at the optimum temperature was then probably similar to that with 40% acetone and no detergent. The state of the substrate may be more reproducible in test mixtures containing a detergent and these may be more suitable for kinetic studies, although not necessarily approximating better to physiological conditions.

Sugar-beet leaves have exceptionally high chlorophyllase activity and the enzyme seems to be extracted in soluble form more readily than from other species, so may not be typical. Comparison of its properties with those of the enzymes from other species is difficult because of the variety of test methods that have been used. Vorob'eva and Krasnovskii[13] studied some properties of sugar-beet chlorophyllase. The only gel-filtration studies are those of Grob and Seiler[14] on the enzyme from *Chlorella pyrenoidosa*; activity seemed to be located mainly in the high-molecular weight fractions, but the buffer concentration was not specified.

[12] M. F. BACON, *Biochem. J.* **101**, 34C (1966).
[13] L. M. VOROB'EVA and A. A. KRASNOVSKII, *Biokhimiya* **31**, 573 (1966).
[14] E. C. GROB and J. SEILER, *Chimia*, **21**, 466 (1967).

Our results show that with a protein such as sugar-beet chlorophyllase, that easily associates with itself or with other substances and adsorbs strongly to negatively charged surfaces, the results of some separation procedures must be interpreted with caution. The various indications of multiple forms of the enzyme might all be explained in terms of reversible associations, or of aggregation, or other alterations during extraction and purification. Although the purification achieved by the procedure described was only 45-fold on a protein basis starting from the citrate–sodium chloride extract it was 500-fold starting from the acetone powder assuming that 80% of the nitrogen was protein. Fractions eluted from Sephadex anion-exchangers had higher specific activities, and it should be possible to obtain greater purification, particularly if the enzyme activity could be stabilised without having to add bovine serum albumin and if the losses at each stage could be diminished.

Increasing the salt concentration of the buffer lessened association of the enzyme and adsorption to negative groups. This may explain why the yield improves when the sodium chloride concentration in the extracting buffer is increased. Adatthody and Racusen[15] consider that insoluble polymers in leaf tissue can act as ion-exchangers and adsorb some soluble proteins unless the buffer concentration is adequately large.

EXPERIMENTAL

Determination of Chlorophyllase Activity

The substrate was prepared by grinding sugar-beet leaves (*Beta vulgaris* var. *saccharifera*) in cold acetone (4 ml/g leaf), leaving the extract at 4° for several days, and filtering off the precipitated carotene.[3] The solution contained about 20% of water and 0·19 mg/ml of chlorophyll *a* and 0·05 mg/ml of chlorophyll *b* as determined by the method of Arnon.[16] For economy we used a crude chlorophyll preparation and have no reason to think that pure chlorophyll would have given different results. At 21° (±1), $(2-x)$ ml of 0·02 M sodium citrate (pH 7·3) were blown into 2 ml of the chlorophyll solution in a 20 ml tube, shaken, and x ml of the enzyme solution was then blown in. The value of x was such that the percentage conversion of chlorophyll *a* to chlorophyllide *a* was usually between 20 and 60 per cent. The mixture was shaken and left in the dark for 30 min. Diethyl ether (2 ml) was added and the mixture was shaken briefly but vigorously. Saturated aq. NaCl (about 14 ml) was added at once, the phases were left to separate in the dark for 5 min, then the contents of the tube were swirled briefly to mix in any pigment at the interface and to break a slight emulsion formed when the protein content was large. The upper layer containing the pigments was sufficiently free from water to be spotted (1–2 μl) directly on 250 μ cellulose plates for TLC, without chlorophyll being retained at the origin.[17] The spot was usually concentrated into a thin line by an initial development with acetone.

The chromatogram was developed in the dark with light petroleum (60–80°)-acetone (80:20 v/v).[11] Chlorophyllides *a* and *b* and chlorophylls *a* and *b* were well separated from each other and the carotenoids, and development for 8 cm on a plate or 6 cm on a microscope slide took only about 6 min. When dry, the chromatogram was held over a 25 W bulb in a lit room so that it was illuminated both from below and above. The percentage conversion of chlorophyll to chlorophyllide was then measured without delay because the pigments soon fade. The sizes and intensities of the chlorophyll *a* and chlorophyllide *a* spots were compared by eye (the *b* pigments being ignored) and an estimate was thereby made of the relative amounts of each pigment. With practice the agreement between duplicate runs and different observers was good, and solutions differing in enzyme activity by about 5% could be distinguished. The method made it possible to measure the activity of many column fractions simultaneously and rapidly on the same TLC plate and was much quicker than eluting the bands and measuring pigment absorption in the spectrophotometer. The extent of conversion given by each fraction could thus be compared directly with that of its neighbours, enabling the positions of peaks of enzyme activity to be located accurately. The percentage conversion was not proportional to enzyme content above about 50% hydrolysis and the results were corrected accordingly.

One unit of chlorophyllase activity is defined as the amount that catalyses the hydrolysis of 1 μmole of the chlorophyll *a* in the substrate to chlorophyllide *a* in 1 min at 21°.

Sodium chloride more concentrated than 0·1 M in the test mixture inhibits chlorophyllase action. Therefore, when fractions containing 0·5 M NaCl did not give sufficient conversion with 0·8 ml test portions, the

[15] K. K. Adatthody and D. R. Racusen, *Can. J. Bot.* **45**, 2237 (1967).
[16] D. I. Arnon, *Plant Physiol. Lancaster* **24**, 1 (1949).
[17] M. F. Bacon, *J. Chromatog.* **17**, 322 (1965).

time of action was increased rather than the amount of enzyme. Bovine serum albumin (BSA) at 0·4 mg/ml increased the conversion to chlorophyllide by about 35 per cent.

Protein

This was measured by nitrogen analysis (micro-Kjeldahl) and by the method of Lowry *et al.*[18] using BSA as standard. It was also estimated from the u.v. absorption at 250, 280 and 310 nm, taking as the corrected value

$$E_{280} - \frac{(E_{250} + E_{310})}{2} \quad (E = \text{extinction at the wavelength indicated measured in a 1 cm cell}).$$

This correction was used to try to minimize the effect of non-protein u.v.-absorbing or light-scattering contaminants in the extracts. The corrected value multiplied by 1·5 gave an approximate measure of the protein content in mg/ml.

Preparation of Crude Extracts from Acetone Powders

Acetone powders were made from sugar-beet leaves by the method used previously.[3,5] The powder was mixed with a solution containing 0·02 M sodium citrate and 0·4 M NaCl (pH 7·0, 8 ml/g powder), and left at 4° overnight. The extract was squeezed out through cotton cloth and the residue rinsed with 0·02 M sodium citrate (pH 7·3, 2 ml/g powder). The combined extracts were centrifuged at 8000 *g* for 10 min at 4° and the pellet discarded. In some experiments NaCl was not included in the extraction medium.

Chromatography

All operations were done at 2–5°. pH values were measured at about 7°. The grades of Sephadex used were: G-100; carboxymethyl-Sephadex, CM-Sephadex C-50; DEAE-Sephadex A-50; diethyl-2-hydroxypropyl ammonium Sephadex, QAE-Sephadex A-50; all 40–120 μ.

Gel-filtration on G-100. G-100 (24 g) was soaked in 0·02 M sodium citrate containing 0·5 M NaCl (pH 7·0) for 2 days and then packed in the column with the same solution. NaCl was omitted in some experiments. The sample was applied in a volume not exceeding 22 ml and effluent volumes were measured from the moment when half the sample had entered the bed. The bed volume was about 375 ml and the flow rate 50 ml/hr. Usually 80–100 per cent of the enzyme activity was recovered; when the loading was very small the recovery was sometimes less but could be improved by adding BSA to each collecting tube. Freshly prepared columns were run with a portion of crude extract before use, to presaturate any protein-absorbing groups on the gel.

The following substances were used for molecular weight calibrations:[19,20] blue dextran 2000 (Pharmacia), bovine albumin (Koch-Light, cryst. puriss.), chymotrypsin (Sigma, cryst. ex bovine pancreas), chymotrypsinogen (Koch-Light, "pure") and ovalbumin (Koch-Light, 5 × cryst.). Elution volumes of these markers from a column of bed volume 384 ml run with 0·02 M citrate—0·5 M NaCl were, respectively, 107, 147, 222, 219 and 178 ml. BSA dimer was eluted at 114 ml.

For small-scale experiments, columns of 1 g of G-100 were packed and developed with either 0·02 M sodium citrate—0·5 M NaCl, pH 7·0 or 0·03 M tris—0·006 M HCl, pH 8·9. The sample was applied in 1 ml and contained 0·3 to 0·6 mg protein.

Ion-exchange on CM-Sephadex. CM-Sephadex was soaked in 0·02 M sodium citrate—0·5 M NaCl for 24 hr and then equilibrated against 0·02 M citrate buffer, pH 5·8 containing 0·1 M NaCl.

For initial concentration and purification, the crude extract from 17 g acetone powder (about 135 ml, pH 7·1) was diluted and adjusted to pH 6·3 by adding 0·2 vol. 0·02 M citric acid and 2·4 vol. distilled water. Traces of precipitate were filtered off under reduced pressure. The filtrate was run through a column containing 3 g CM-Sephadex (bed vol. 100 ml, flow rate about 200 ml/hr). The column was washed with 150 ml 0·02 M citrate—0·1 M NaCl buffer (pH 5·8) and the retained chlorophyllase was then eluted with 0·02 M sodium citrate—0·5 M NaCl (pH 7·0). The enzyme emerged as a single peak in a total volume of about 80 ml. The most active fractions were pooled (50 ml) and contained about 90 per cent of the retained activity.

For further purification on CM-Sephadex (following an intermediate gel-filtration run on G-100) the preparation was ultrafiltered overnight with dialysis against 0·02 M sodium citrate, pH 7·3. The concentrate was adjusted to pH 5·8 with 0·02 M citric acid, centrifuged and the supernatant (about 10 ml) applied to a column of the ion-exchanger (2 g, bed vol. 60 ml). The column was developed initially with 0·02 M citrate buffer (pH 5·8) containing 0·1 M NaCl, then with a linear gradient of NaCl in the same buffer (final pH 5·4) at about 30 ml/hr. About 80 per cent of the activity was recovered.

Ion-exchange on QAE- and DEAE-Sephadex. The enzyme-containing sample was concentrated by ultrafiltration, with dialysis against 0·025 M tris–0·005 M HCl, pH 8·9, then further dialysed against fresh buffer.

[18] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1957).
[19] P. ANDREWS, *Biochem. J.* **91**, 222 (1964).
[20] P. ANDREWS, *Biochem. J.* **96**, 595 (1965).

After centrifuging, 2 ml of the supernatant fluid, containing 2 mg or less of protein, was applied to a column of the anion-exchanger (0·9 g, bed vol. 50 ml). The column was developed initially with tris-HCl and then with a linear gradient of NaCl in the same buffer. The flow rate was 60 ml/hr; more than 80% of the activity was recovered.

Ultrafiltration and Dialysis

Solutions were ultrafiltered through Visking tubing (¼ in., Scientific Instrument Centre Ltd, London) suspended in evacuated Buchner flasks at 2–5°, essentially as described by Sober *et al.*[21] The open end of the tubing passed through a hole in the rubber stopper of the flask and was wedged in place with a plastic funnel containing the sample. The filtration rate was about 4 ml/hr with an 18 cm sac.

For simultaneous dialysis, the flask was two-thirds filled with the dialysing buffer so that the bottom one-third of the sac was submerged. For further dialysis, the sac was cut and knotted and stirred in fresh buffer. Exchange into buffers of low ionic strength using Sephadex G-25 was not practicable because some of the chlorophyllase was retained by negative groups on the dextran gel.

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[21] H. A. Sober, F. J. Gutter, M. M. Wyckoff and E. A. Peterson, *J. Am. chem. Soc.* **78**, 756 (1956).