Acetolactate Synthase from Barley (*Hordeum vulgare* L.): Purification and Partial Characterization

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Acetolactate Synthase (ALS), Acetohydroxy Acid Synthase (AHAS), Amino Acids (Branched-Chain), *Hordeum vulgare* L., Feedback Inhibition

Acetolactate synthase (EC 4.1.3.18; ALS) has been extracted from etiolated barley shoots (*Hordeum vulgare* L.) and purified to near homogeneity. Purification was made possible by a five-step procedure using hydrophobic interaction, gel filtration, anion-exchange and hydroxylapatite chromatography, the last two steps performed with an HPLC- and FPLC-system, respectively. A 300-fold purification was achieved representing 13% of the initial activity in the crude extract; only small amounts of pure acetolactate synthase could be isolated. Although the enzyme was found labile during the chromatographic steps, purified ALS maintained its activity for several hours and could be stored at 70 K for weeks with a 15–30% loss.

The apparent molecular weights of the enzymatically active species as determined by gel filtration were about 440 kDa and 200 kDa, respectively. We assume these species are no isozymes but different polymeric forms of a basic unit of ALS. SDS-PAGE analysis showed one polypeptide with an apparent molecular weight of 58 kDa. Preliminary enzymatic characterization of the purified enzyme confirms a marked synergism in the feedback control by branched-chain amino acids. The combination of valine plus leucine exhibited the most co-operative inhibition.

Introduction

Acetolactate synthase (ALS; also referred as acetohydroxy acid synthase, AHAS) catalyzes the first common step in the biosynthetic pathway of the essential branched-chain amino acids valine, leucine and isoleucine. The enzyme can either catalyze the formation of acetohydroxybutyrate from pyruvate and α-oxobutyrate or the synthesis of acetolactate from two molecules of pyruvate. ALS has been found in enteric bacteria [1–3], mitochondria of fungi [4], and in chloroplasts of higher plants [5]. The enzyme has raised strong interest since three structurally unrelated classes of herbicides, the sulfonylureas [6], imidazolinones [7], and triazolo pyrimidines [8] have been shown to specifically inhibit ALS. These compounds exhibit strong herbicidal potency combined with low mammalian toxicity probably due to the fact mammalians lacking ALS (but compare ref. [9]).

Genes coding for three ALS isozymes (I, II and III) have been determined in *Salmonella typhimurium* and *Escherichia coli* [10–13]. In these bacteria ALS is a tetramer composed of two large and two small subunits in an $\alpha_2\beta_2$-structure, with apparent molecular weights of 60 kDa and 9 to 17 kDa respectively [1–3]*. Little is known on plant enzymes about structure, subunits, specificity and herbicidal interaction. In the yeast *Saccharomyces cervisiae* only one functional ALS gene could be found [14]. Recently, one ALS gene from *Arabidopsis thaliana* and *Nicotiana tabacum* have been identified and isolated, respectively [15]. Due to extreme lability none of the eukaryotic ALS enzymes has been purified yet, although some progress has been made by partially purifying ALS from *Neurospora crassa* [16] and *Zea mays* [17]. In this paper a procedure to obtain ALS with a high specific activity from barley is presented and some biochemical details are given. This report is a step to further studies on the enzymology of acetolactate synthase.

Materials and Methods

Plant material

Seeds of barley (*Hordeum vulgare* L.) were placed onto a nylon net, covered with a filter paper, wetted

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* Apparent molecular weights are expressed as kDa whether they have been determined by SDS-PAGE or gel filtration.

with distilled water and allowed to germinate in a dark chamber at 23–26 °C. After germinating (24–36 h), roots were immersed into a medium containing 2 g/l KNO_3, 34 mg/l $CaSO_4$ and 0.25 mg/l Na_2MoO_4, pH 7.0. Three days after germinating etiolated shoots were harvested, washed in distilled water, collected and stored at 70 K for later use. Green barley shoots were cultivated accordingly under daylight in the greenhouse and harvested 6 days after germinating. Chlorophyll was 0.7 to 1.1 mg/g fresh weight.

Assays

Unless otherwise indicated, the standard assay mixture contained 32 mM Na-phosphate, pH 6.5, 40 mM Na-pyruvate, 0.5 mM thiamine pyrophosphate (TPP), 1 mM $MnCl_2$, 50 μM flavin adenine dinucleotide (FAD) plus enzyme in a final volume of 0.5 ml. After incubation for 30 min at 30 °C, the reaction was stopped with 0.25 ml 3 M H_2SO_4. The tubes were heated at 60 °C for 12 min and acetoin was determined by the method of Westerfeld [18]. Under these conditions 100 nmol/1.3 ml acetoin were equivalent to an absorbance of 1.55 at 530 nm.

Protein was determined according to Bradford [19].

Enzyme purification

All operations were carried out at 4 °C. Light exposure during chromatography was minimized by covering columns, capillaries and fraction collectors with aluminum foil. Frozen shoots, usually 100–150 g, were homogenized in 400–600 ml of standard buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA and 15% (v/v) glycerol) containing 1 mM L-leu, 1 mM L-val, 0.1 mM FAD and 1 mM dithiothreitol (DTT). The shoots were homogenized in a blender for 4 times each sample with a 10–20 seconds period. Cell debris was removed by centrifugation for 7 min at 20,000 × *g*. Solid ammonium sulfate was added to the supernatant (30% saturation, 1.76 g/10 ml) and the precipitate collected by centrifugation for 15 min at 27,000 × *g*. The supernatant was dialyzed against in standard buffer containing additions as indicated as well as 1 M ammonium sulfate and applied to a column of butyl-sepharose CL-4B (3.2 × 7 cm). Elution was performed by a linear gradient consisting of 200 ml of the medium just mentioned and 200 ml buffer without ammonium sulfate at a flow of 3 ml/min. Fractions containing ALS activity were pooled, concentrated by precipitation using ammonium sulfate with 45% saturation (2.76 g/10 ml) and centrifugation as described above. The precipitate was resuspended in standard buffer and desalted on Sephadex G-25 PD-10 minicolumns equilibrated with standard buffer containing 1 mM DTT and 10 mM pyruvate. The desalted protein was bound to Fractogel TSK DEAE-650 (medium pressure, 2 × 8 cm column). After washing with 60 ml of starting buffer ALS was eluted using an NaCl gradient (200 ml from zero to 250 mM) at a flow rate of 2 ml/min. Fractions containing ALS activity were pooled and concentrated by ultrafiltration using an Amicon-cell with a PM-30 membrane. The protein was placed on a gel filtration column (Bio-Gel A-1.5, 2.7 × 90 cm) equilibrated with standard buffer containing 1 mM DTT, 10 mM pyruvate, 10 μM FAD and 25 mM NaCl with a flow rate of 0.3 ml/min. Fractions containing ALS activity were subsequently loaded on an anion-exchange HPLC-column (Bio-Gel TSK DEAE-5-PW). Again ALS was eluted by an NaCl gradient (30 min, 25 mM to 250 mM) with the buffer as used for the DEAE-650 column. Active fractions were desalted on PD-10 minicolumns equilibrated with 25 mM Na-phosphate, pH 7.5, 5 mM $MgCl_2$, 3 mM DTT, 10 μM $CaCl_2$ and 10% (v/v) glycerol, applied on a hydroxylapatite HPLC-column (Bio-Gel HPHT) and eluted with a Na-phosphate gradient (40 min, 25 mM to 200 mM, pH 7.5). During the HPLC-steps temperature was 4 °C and the operating pressure below 210 psi (14 bar).

Polyacrylamide gel electrophoresis (SDS-PAGE)

Our technique based on the method of Lämmli [20]; polyacrylamide was 17.5% (w/w) for the separating and 5% for the stacking gel. The separating gel contained 4 M urea. Gels were stained with Coomassie brilliant blue R 250 and/or with silver nitrate.

Molecular weight determination

The apparent molecular weight was estimated with a FPLC-system equipped with semi-preparative columns of Superose 12 (1.6 × 60 cm, 6 prep grade). Both columns were serially connected. Buffer was 100 mM Tris/HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM EDTA, 50 mM NaCl and 10% (v/v) glycerol.

Flow rates were 1 or 0.3 ml/min. Molecular weight markers from 29 to 700 kDa were purchased from Sigma, Munich.

Fine chemicals

Butyl-sepharose CL-4B, Superose 6 and 12 prep grade and Sephadex G 25 (PD-10) minicolumns were purchased from Pharmacia Fine Chemical Inc., Freiburg. Bio-Gel A-1.5 M and the HPLC-columns Bio-Gel TSK DEAE-5-PW and Bio-Gel HPHT are products of Bio-Rad Laboratories, Munich. Flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), dithiothreitol (DTT), amino acids and molecular weight markers for SDS-PAGE were obtained from Sigma Chemical Co., Munich. Fractogel TSK DEAE-650 M was purchased from Merck, Darmstadt, acetoin (3-hydroxy-2-butanone) from Aldrich-Chemie, Steinheim.

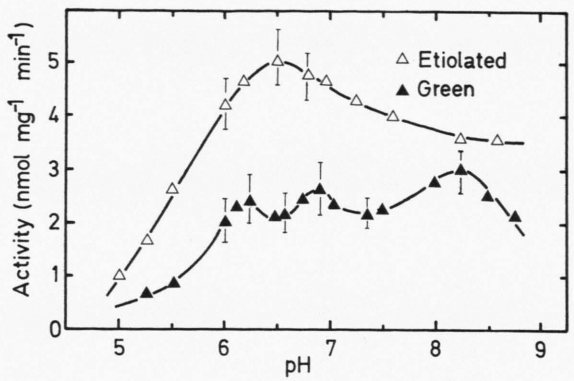

Fig. 1. Effect of pH on ALS activity in crude extracts from etiolated (△) and green barley shoots cultivated under light (▲). Some data carry bars to indicate the standard deviation from three experiments found similar for all points of the curves.

Results

Several different homogenization procedures were tried to maximize the yield of enzyme from etiolated barley shoots. Homogenization of fresh shoots by grinding with sea sand for 5 min gave the highest yield of ALS activity, referred either to specific activity or fresh weight. Due to the small samples which could be homogenized this way (20–40 g), frozen shoots were homogenized in a Waring-blender as the method of choice.

Fig. 1 allows for a comparison of pH-dependence of ALS in crude extracts from etiolated shoots and shoots grown under light. Although the specific ALS activity measured at pH 6.5 differed from 4 to 6.5 nmol min^{-1} mg^{-1} protein depending on the different preparations the course of activity was identical over the pH range as indicated. In the etiolated preparation there was a broad optimum at pH 6.5, while in "green" barley extracts three distinct optima between pH 6 and pH 8.5 were evident. Assays with green soybean cotyledons led to similar findings (data not shown).

The results of an average purification are presented in Table I. An affinity chromatography step on a leucine-agarose column is not documented. Such a column was used between the gel filtration and the DEAE-HPLC column as a further purification step in case of more than 150 g shoots had been homogenized. Binding of ALS to leucine-agarose was performed in 750 mM Na-phosphate, pH 6.5, eluting in 500 mM Na-phosphate, pH 7.5; additions as described for the hydroxylapatite chromatography were added.

The specific activity eventually attained was more than 1500 nmol acetolactate formed per mg protein per minute (nmol min^{-1} mg^{-1}), representing a 300-fold purification. The overall yield of activity of this

Table I. Purification of acetolactate synthase from barley (*Hordeum vulgare* L.).

Step	Protein [mg]	Specific Activity [nmol min^{-1} mg^{-1}]	Yield [%]	Lane No. used in Fig. 3
30–45% $(NH_4)_2SO_4$*	650	5.5	(100)	
Fractogel DEAE	45	60.5	74	
Gel filtration	3	350	28	(1)
DEAE-TSK**	0.8	810	17	(2)
Hydroxylapatite**	0.3	1590	13	(3)

* Including the butyl-sepharose CL-4B column (see text).
** HPLC/FPLC-technique.

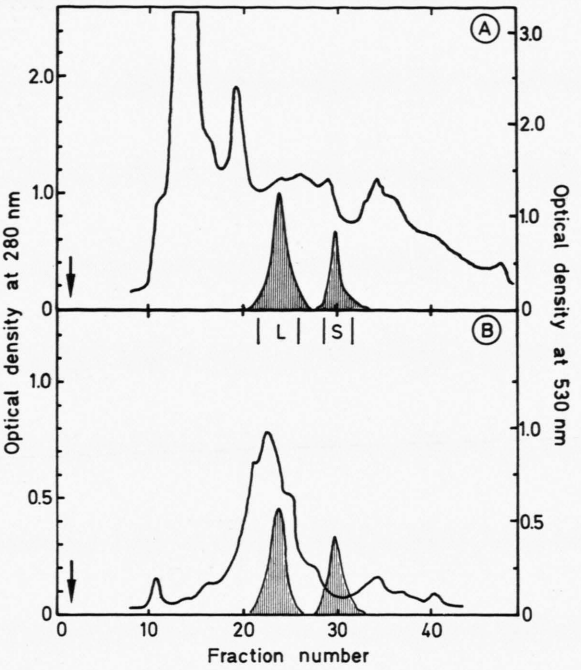

Fig. 2. Elution profile of barley ALS (etiolated shoots) from gel filtration (Superose 6 and 12 prep grade, serially connected columns, FPLC-technique). (A) Chromatography of concentrated DEAE-sepharose fraction. ALS activity is plotted as absorbance at 530 nm (standard assay, right ordinate) resulting in the hatched peaks 'L' and 'S'. Protein was measured by absorption at 280 nm (left ordinate). (B) Re-chromatography of the pooled fractions of peak 'L' from the first run of (A). Elution conditions and columns are identical to (A).

laborious 5-step procedure was 13%. Fig. 2 shows a typical elution profile for molecular weight estimation on Superose (FPLC-system), also characteristic for a preparative gel filtration on Bio Gel A-1,5 applied as the third step in the purification procedure (Table I). The 'S' peak had to be discarded since pooling of the two fractions 'L' and 'S' reduced the efficiency of the gel filtration column. However, a drastic loss of activity had to be accepted.

While loss of activity of crude extracts in the presence of 50 μM FAD and 20% glycerol was 20–25% only when stored at 70 K, purification was hampered by an extreme lability during chromatographic steps, particularly evident by anion-exchange chromatography and binding to leucine-agarose. Stabilizing the enzyme by adding cofactors and substrates had no effect. Purified ALS was stored at 70 K in standard buffer containing 0.1 mM FAD with a loss of activity of 15 to 30% after 15–25 days.

The apparent molecular weight of ALS was determined by gel filtration chromatography on serially connected Superose columns (6 and 12 prep grade, FPLC-system) as described above. In run A (Fig. 2A) a sample obtained by the first anion-exchange step was used. The activity profile resolved two peaks. Freezing and thawing of the large fraction ('L') and subsequent re-chromatography on the same columns again resolved both fractions 'L' and 'S'. A similar activity profile by re-chromatography was obtained by an increased salt concentration (100 mM NaCl) of the running buffer. Re-chromatography of the 'S'-fraction led tof the 'S'-peak only, a reconstitution of a 'L'-peak could not be observed. Molecular weights of each species were determined as approximately 430 to 460 kDa ('L') and 190 to 210 kDa ('S') using standard marker proteins.

The protein pattern resolved by SDS-gel electrophoresis at different stages of purification are shown in Fig. 3. The last purification step on hydroxylapatite (HPHT-column; HPLC-technique) led to a 58-kDa band on the SDS-PAGE, without 4 M urea a band of 59 kDa was observed.

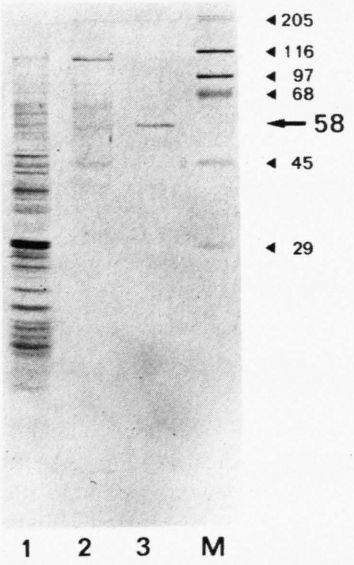

Fig. 3. SDS-PAGE of subsequent purification steps of ALS from etiolated barley shoots (see Table I). Lane 1, gel filtration; lane 2, DEAE-TSK (HPLC technique); lane 3, hydroxylapatite (HPLC). Samples loaded in lane 1–3 were equivalent to an activity of 1.5 nmol acetoin/min, the sample of lane 3 was equivalent to 3 nmol/min. Molecular weight markers are indicated by the right lane (M).

SDS-PAGE with parallel silver staining was performed with consecutive fractions of the ALS activity peak obtained by the last purification step on hydroxylapatite (Fig. 4A). Fig. 4B correlates the peak-area of the 58-kDa band, measured by laser densitometry, and the corresponding ALS activity of the samples. The diffuse bands in the 50- to 60-kDa range have been commonly observed in silver-stained gels and shown to be artificial [21]. Obvious is the correlation of ALS activity and densitometrically estimated amount of protein of the 58-kDa band.

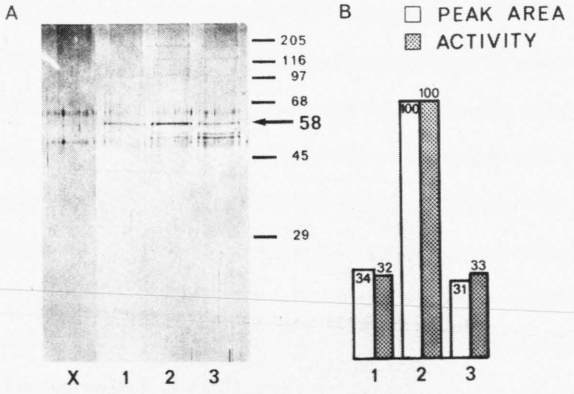

Fig. 4. SDS-PAGE of fractions from the last purification step of barley ALS on hydroxylapatite (lane 1 to 3). The position of molecular weight markers are noted at the right of the figure (A). The sample of lane X shows the weak bands only obtained with the buffer used for hydroxylapatite chromatography as reported [21]. After silver staining lanes 1–3 were scanned with a laser densitometer (Ultrascan 2202, LKB, Bromma, Sweden). The peak areas of the 58-kDa band and the corresponding activities of the samples loaded on the gel are shown in (B). The activity loaded on lane 2 was 2.1 nmol acetoin formed/min, which was taken as 100%.

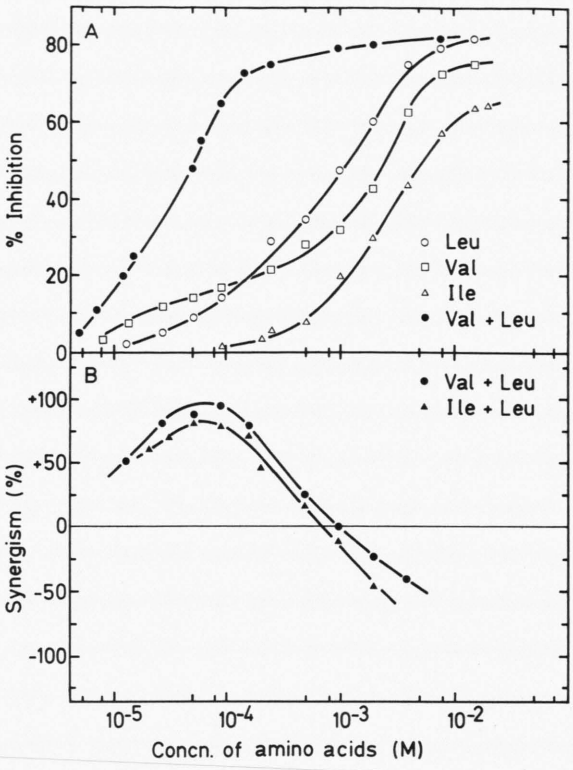

Fig. 5. Inhibition of barley ALS by branched-chain amino acids. (A) Percentage inhibition plotted against increasing concentrations of amino acids. In case of combination of valine + leucine or leucine + isoleucine (not shown in A) molar concentrations were 1:1. Specific activities of the enzyme preparations used were between 1.0 and 1.3 μmol mg^{-1} min^{-1}. (B) Synergistic inhibition of valine + leucine and isoleucine + leucine calculated from the data shown in (A). Percentage synergism is expressed by the ratio of the measured inhibition by the amino acids combined divided by the calculated sum of the individual inhibition by each amino acid.

The influence of branched-chain amino acids on ALS activity is shown in Fig. 5A. Inhibition was observed with leucine, valine and to a lesser extent with isoleucine. At concentrations higher than 0.1 mM leucine and at concentrations below 0.1 mM valine were the most potent inhibitors. The inhibition by leucine plus valine combined was more efficient than caused by each amino acid alone. The synergistic effect of valine plus leucine or isoleucine plus leucine was evident between 50 and 100 μM of each amino acid. These combinations exhibited a synergistic effect of nearly 100%, *i.e.* twice as high inhibition as compared with the calculated sum of the single effects (Fig. 5B). The combination valine plus isoleucine was negligible (not shown). A second evidence for co-operative inhibition is the shift of the I_{50} value. While the corresponding values of valine and leucine are 1.1 mM and 2.0 mM, respectively, the combination of both showed a more than 10-fold inhibitor potency ($I_{50} = 110$ μM; 55 μM of each amino acid present in the assay).

A strict Michaelis-Menten kinetics of ALS with respect to pyruvate was observed. Even at concentrations below 1 mM pyruvate no sigmoidal shape became apparent. Maximum activity was reached at

approximately 50 mM pyruvate, the K_M for pyruvate was found at 5.5 mM (date not shown).

Discussion

In ripening pea seeds the existence of two ALS isozymes was assumed operating at pH 6 to 7 and pH 8 to 8.5, respectively [22]. Also with etiolated barley shoots two pH-optima between pH 6 and 7 were observed [23]. The pH-dependent activities of etiolated and green barley documented in Fig. 1 seem to confirm these findings, although data with crude extracts need careful interpretation. For example, the single pH-optimum of the etiolated preparation may be composed of two pH-optima, but of different height as observed between pH 6 and 7 in crude extract of green barley. Etiolated barley was taken as the most convenient enzyme source having less complexity of pH-dependence. Furthermore, the etiolated shoots yielded twice as much ALS either referred to protein or fresh weight as compared to green barley.

The elution pattern of ALS activity with two active fractions ('L' and 'S') obtained by gel filtration (Fig. 2) shows a second feature with respect to possible ALS isozymes. Splitting into two active peaks during column chromatography has been reported recently with corn ALS [24]. Furthermore, there is genetic evidence for multiple forms of ALS in *Nicotiana tabacum* [25]. The re-chromatography as applied in this study and shown in Fig. 2B indicates the two ALS peaks as at least partly due to different polymeric states of a basic ALS unit. Whether active fractions obtained by column chromatography represent true ALS isozymes or are aggregates induced by chromatography conditions requires further studies.

Surprisingly, SDS-PAGE of the purified enzyme leads to a single 58-kDa band. Reports on bacterial ALS (1–3) claimed the enzyme to be composed of large (59 to 60 kDa) and small (9 to 17 kDa) subunits. Admittedly, lost of a small subunit due to our preparation conditions, though impropable, cannot be excluded, the same holds true for a possible poor staining of this polypeptide thereby escaping detection. The ALS genes from *Arabidopsis thaliana* and *Nicotiana tabacum* have been demonstrated to be homologous with the genes encoding the large bacterial subunit [15]. Neither in these plants nor in yeast [14] a sequence encoding a smaller subunit has been found.

The findings on subunit composition of native plant ALS are puzzling. The 440-kDa peak ('L'), observed also with corn ALS [17], may be a dimer of the smaller active species ('S' in our elution pattern) with a molecular weight of 200 kDa. Whether this smaller species is composed of three or four 58-kDa subunits is still unclear.

Older studies on the barley enzyme using concentrated crude extracts demonstrated feedback and co-operative inhibition by valine, leucine and isoleucine [23]. Only the purified ALS allowed us to assay amino acid concentrations below 0.1 mM. Both, the sigmoidal inhibition of ALS and valine being the most potent inhibitor below 0.1 mM could be shown for the first time (Fig. 5). Our studies on herbicidal inhibition exhibited identical I_{50} values for chlorsulfuron (33 nM; 30 min assay, see ref. [6]) for both crude extracts and purified ALS (data not shown).

At present, further characterization and inhibition studies are hindered by the small quantities of purified enzyme. We are confident, however, to upscale this procedure in due time.

Acknowledgements

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[1] L. Eoyang and P. M. Silverman, J. Bacteriol. **157,** 1184–1189 (1984).
[2] H. Grimminger and H. E. Umbarger, J. Bacteriol. **137,** 846–853 (1979).
[3] J. V. Schloss, D. E. Van Dyk, J. V. Vasta, and R. M. Kutny, Biochemistry **24,** 4952–4959 (1985).
[4] E. D. Ryan and G. B. Kohlhaw, J. Bacteriol. **120,** 631–637 (1974).
[5] A. V. Jones, R. M. Young, and K. Leto, Plant Physiol. **77,** S-293 (1985).
[6] T. R. Ray, Plant Physiol. **75,** 827–831 (1984).
[7] D. L. Shaner, P. C. Anderson, and M. A. Stidham, Plant Physiol. **76,** 545–546 (1984).
[8] W. A. Kleschick, Eur. Pat. Applic. **142152** (1984); comp. J. V. Schloss, L. M. Ciskanik, and D. E. Van Dyk, Nature **331,** 360–362 (1988).
[9] S. Matsunaka, N. Nakata, K. Hioki, Y. Noguchi, and O. Yoshitake, Proc. Brit. Crop Prot. Conf. (Weeds), **Vol. 1,** 139–145, BCPC-Publ., Croydon U.K. 1985.
[10] P. Friden, J. Donegan, J. Mullen, P. Tsui, M. Freundlich, L. Eoyang, R. Weber, and P. M. Silverman, Nucleic Acids Res. **13,** 3979–3993 (1985).
[11] R. P. Lawther, D. H. Calhoun, C. W. Adams, C. A. Hauser, J. Gray, and G. W. Hatfield, Proc. Natl. Acad. Sci. U.S.A. **78,** 922–925 (1981).
[12] C. H. Squires, M. DeFelice, J. Devereux, and J. M. Calvo, Nucleic Acids Res. **11,** 5299–5313 (1983).
[13] R. C. Wek, C. A. Hauser, and G. W. Hatfield, Nucleic Acids Res. **13,** 3995–4010 (1985).
[14] S. C. Falco and K. D. Dumas, Genetics **109,** 21–35 (1985).
[15] B. J. Mazur, C. F. Chui, and J. K. Smith, Plant Physiol. **85,** 1110–1117 (1987).
[16] H. Tanaka and H. Kuwana, Biochem. Biophys. Res. Commun. **123,** 418–423 (1984).
[17] M. J. Muhitch, Plant Physiol. **86,** 23–27 (1988).
[18] W. W. Westerfeld, J. Biol. Chem. **161,** 495–502 (1945).
[19] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[20] U. K. Laemmli, Nature **227**, 680–685 (1970).
[21] D. Ochs, Anal. Biochem. **135,** 470–474 (1983).
[22] M. E. Davies, Plant Physiol. **39,** 53–59 (1964).
[23] M. J. Miflin, Arch. Biochem. Biophys. **146,** 542–550 (1971).
[24] M. J. Muhitch, D. L. Shaner, and M. A. Stidham, Plant Physiol. **83,** 451–456 (1987).
[25] R. S. Chaleff and N. F. Bascomb, Mol. Gen. Genet. **210,** 33–38 (1987).