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**Update section**

*Short communication*

# Wheat acetyl-CoA carboxylase

Piotr Gornicki and Robert Haselkorn *
*Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, USA*
*(* author for correspondence)*

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**Abstract**

The acetyl-CoA carboxylase present in both wheat germ and total wheat leaf protein contains ca. 220 kDa subunits. It is the major biotin-dependent carboxylase present in wheat chloroplasts. Active acetyl-CoA carboxylase purified from wheat germ is a homodimer with an apparent molecular mass of ca. 500 kDa. The enzyme from wheat germ or from wheat chloroplasts is sensitive to the herbicide haloxyfop at micromolar levels. The incorporation of $^{14}$C-acetate into fatty acids in freshly cut wheat seedling leaves provides a convenient *in vivo* assay for both acetyl-CoA carboxylase and haloxyfop.

Acetyl-CoA carboxylase (ACC) is the first enzyme of the biosynthetic pathway to fatty acids. It belongs to a group of carboxylases that use biotin as cofactor and bicarbonate as a source of the carboxyl group. *Escherichia coli* ACC contains three functional domains on four distinct, separable polypeptides: biotin carboxylase (BC) is a dimer of 49 kDa monomers, biotin carboxyl carrier protein (BCCP) is a dimer of 17 kDa monomers and transcarboxylase (TC) is a tetramer containing two each of 33 kDa and 35 kDa subunits. The primary structures of all of the *E. coli* ACC subunits and the biotin carboxylase and biotin carboxyl carrier proteins of *Anabaena* 7120 are known, based on the gene sequences [2, 8, 9, 14; P.Gornicki and R.Haselkorn, unpublished].

Yeast, rat and chicken ACC are cytoplasmic enzymes consisting of 250 to 280 kDa subunits that contain all of the bacterial enzyme activities. Their primary structure has been deduced from cDNA sequences [1, 11, 17]. Animal ACC content and/or activity varies with the rate of fatty acid synthesis or energy requirements in different nutritional, hormonal and developmental states. In the rat, ACC mRNA is transcribed using different promoters in different tissues and can be regulated by alternative splicing. The rat enzyme activity is also regulated allosterically by a number of metabolites and by reversible phosphorylation [e.g. 12, 18]. The wheat enzyme, as well as those from other plants, is composed of ca. 220 kDa subunits, similar to the enzyme from other eukaryotes [3, 4, 6, 16, 19]. Plant ACC is located in the stroma of plastids, the primary site of plant fatty acid synthesis. The gene must be nuclear because no corresponding sequence has been seen in the complete chloroplast DNA sequences of tobacco, liverwort or rice. ACC, like the vast majority of chloroplast proteins that are encoded in nuclear DNA, must be synthesized in

the cytoplasm and then transported into the chloroplast.

Although the basic features of plant ACC must be the same as those of prokaryotic and other eukaryotic ACCs, significant differences can also be expected due, for example, to differences in plant cell metabolism and ACC cellular localization. We have recently cloned fragments of wheat ACC cDNA [P.Gornicki and R.Haselkorn, unpublished results]. Peptide sequences deduced from the cDNA sequences (ca. 1.5 kb) showed high identity with yeast, rat and chicken ACC, especially within the BC domain. In this paper, analysis of the wheat protein structure and the enzyme inhibition by haloxyfop, one of the herbicides that target ACC, are reported.

Biotin-containing (streptavidin-binding) proteins in extracts prepared from leaves of two-week old seedlings of wheat and pea, both total protein and protein from intact chloroplasts (prepared by centrifugation on Percoll gradients as described previously [5]), and from wheat germ (Sephadex G-100 fraction prepared as described below) were analyzed by western blotting with $^{35}$S-Streptavidin (Fig. 1). Proteins were separated by SDS-PAGE using a 7.5% separating gel (13), and then were transferred onto a PVDF membrane (Immobilon-P, Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11), 10% methanol, at 4 °C, 40 V, overnight. The blots were blocked with 3% BSA solution in 10 mM Tris-HCl pH 7.5 and 0.9% NaCl and then incubated for 3–16 h with $^{35}$S-Streptavidin (Amersham). The blots were washed at room temperature with 0.5% Nonidet-P40 in 10 mM Tris-HCl pH 7.5 and 0.9% NaCl.

In wheat, the 220 kDa protein was present in both total and chloroplast protein. It was the major biotinylated polypeptide in the chloroplast protein (traces of smaller biotinylated polypeptides, most likely degradation products of the large one, could also be detected). ACC consisting of 220 kDa subunits is the most abundant biotin-dependent carboxylase present in wheat chloroplasts. In pea chloroplasts the biotinylated peptides are much smaller, probably due to greater degradation of the 220 kDa peptide, which could be detected only in trace amounts in some chloroplast preparations (not shown). The amount of all biotinylated peptides, estimated from band intensities on western blots (amount of protein loaded was normalized for chlorophyll content), is much higher in pea than in wheat chloroplasts (Fig. 1).

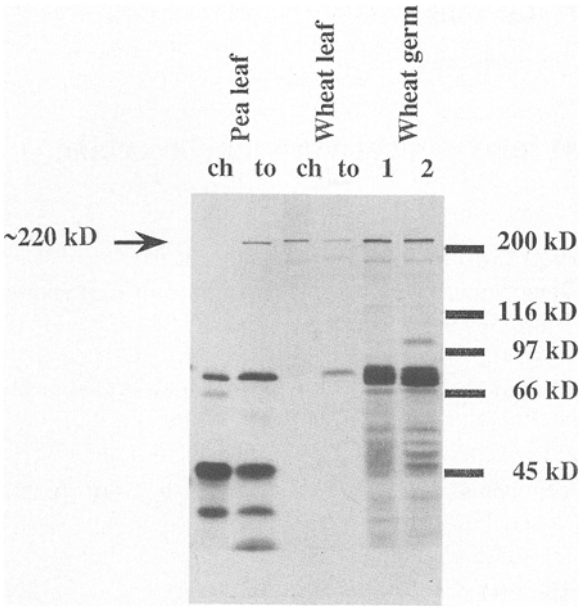


*Fig. 1.* Western analysis of biotinylated peptides in wheat and pea leaves (to, total leaf protein; ch, protein from gradient-purified intact chloroplasts), and in wheat germ (1, wheat germ extract purified through gel filtration on Sephadex G-100 as described in this paper; 2, wheat germ extract purchased from Amersham). $^{14}$C-Streptavidin was used to reveal biotinylated peptides. Location of molecular weight markers is shown on the right.

Purification of wheat germ ACC was carried out at 4 °C or on ice. 200 g of wheat germ (Sigma) were homogenized (10 pulses, 10 s each) in a Waring blender with 300 ml of 100 mM Tris-HCl pH 7.5, 7 mM 2-mercaptoethanol. Two 0.3 ml aliquots of fresh 0.2 M solution of phenylmethylsulfonyl fluoride (Sigma) in 100% ethanol were added immediately before and after homogenization. Soluble protein was recovered by centrifugation for 30 min at 12000 rpm. 1/33 volume of 10% poly(ethyleneimine) solution (pH 7.5) was added slowly and the mixture was stirred for 30 min [4], followed by centrifugation for 30 min

at 12000 rpm to remove the precipitate. ACC in the supernatant was precipitated by adding solid ammonium sulfate to 50% saturation. The precipitate was collected by centrifugation for 30 min at 12000 rpm, dissolved in 200 ml of 100 mM KCl, 20 mM Tris-HCl pH 7.5, 20% glycerol, 7 mM 2-mercaptoethanol, mixed with 0.2 ml of phenylmethylsulfonyl fluoride solution (as above) and loaded on a 5 cm x 50 cm Sephadex G-100 column equilibrated and eluted with the same buffer. Fractions containing ACC activity (assayed as described below using up to 20 $\mu$l aliquots of column fractions) were pooled and loaded immediately on a 2.5 cm x 40 cm DEAE-cellulose column also equilibrated with the same buffer. The column was washed with 500, 250 and 250 ml of the same buffer containing 150, 200 and 250 mM KCl, respectively. Most of the ACC activity was eluted in the last wash. Protein present in this fraction was precipitated with ammonium sulfate (50% saturation), dissolved in a small volume of 100 mM KCl, 20 mM Tris-HCl pH 7.5, 5% glycerol, 7 mM 2-mercaptoethanol, and separated in several portions on two Superose columns connected in-line (Superose 6 and 12, Pharmacia). 1 ml fractions were collected at 0.4 ml/min flow rate. Molecular mass standards were thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; albumin, 67 kDa (Pharmacia). ACC-containing fractions were concentrated using Centricon-100 concentrators (Amicon) and the proteins were separated by SDS-PAGE as described above.

The results of the gel filtration step are shown in Fig. 2. Active ACC has an apparent molecular mass of ca. 500 kDa and the individual polypeptides have a molecular mass of 220 kDa. The 220 kDa polypeptide was the major component of this preparation (fraction 21 and 22 of Fig. 2) as revealed by Coomassie staining of proteins separated by SDS-PAGE (not shown). This preparation also contained several smaller biotin-containing peptides as revealed by western blotting with $^{35}$S-Streptavidin (not shown), most likely degradation products of the ca. 220 kDa peptide, which retained their ability to form the ca. 500 kDa complex and therefore co-purified

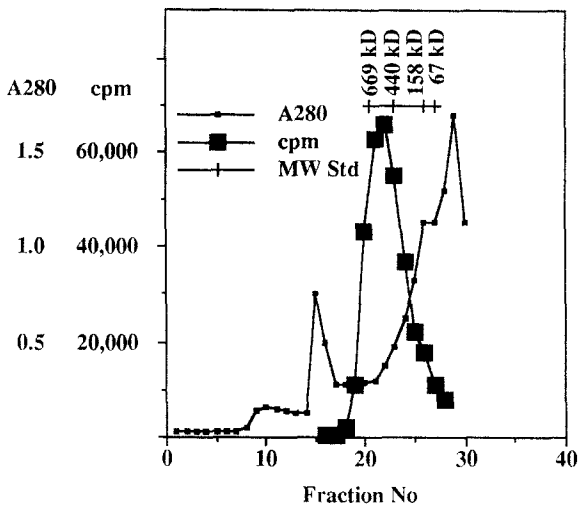


*Fig. 2.* Purification of wheat germ ACC by gel filtration on Superose.

with intact ACC. The ACC preparations were active only when they contained intact 220 kDa biotinylated polypeptide. It is not possible to estimate the recovery of the active ACC, due to continuous degradation of the 220 kDa peptide during purification and to increased recovery of ACC activity in more purified preparations, probably due to separation of the enzyme from inhibitors in the cruder extracts.

The 220 kDA wheat peptide isolated as a dimer according to the above protocol was finally purified by SDS-PAGE and transferred to Immobilon-P for sequencing (as described above). The N-terminus of the peptide appeared to be blocked. A mixture of amino acids was detected only after the protein was cleaved chemically with CNBr. The 220 kDa protein was therefore purified on an SDS gel, cleaved with CNBr, and the resulting peptides were fractionated by gel electrophoresis basically as described [7], with the following modifications. A slice of gel containing about 20 $\mu$g of the 220 kDa polypeptide was dried under vacuum to about half of its original volume and then incubated overnight in 0.5 ml of 70% formic acid containing 25 mg of CNBr. The gel slice was dried again under vacuum to about half of its original volume and was equilibrated in 1 ml of 1 M Tris-HCl (pH 8.0). The CNBr peptides were

separated by inserting the gel piece directly into a well of a tricine gel (as described in [7] but without a spacer gel). Gels used to separate peptides for sequencing were pre-run for 30 min with 0.1 mM thioglycolic acid in the cathode buffer. Peptides were transferred to Immobilon-P for sequencing by the Edman degradation method as described above.

Several bands of peptides, ranging in size from 4 to 16 kDa, with a well-resolved single band at about 14 kDa, were obtained. Attempts to sequence the smaller peptides failed, but the 14 kDa peptide yielded a clean result for residues 3–13. A very similar sequence motif, which includes the presumptive methionine cleaved by CNBr, was found near the C-terminus of published sequences

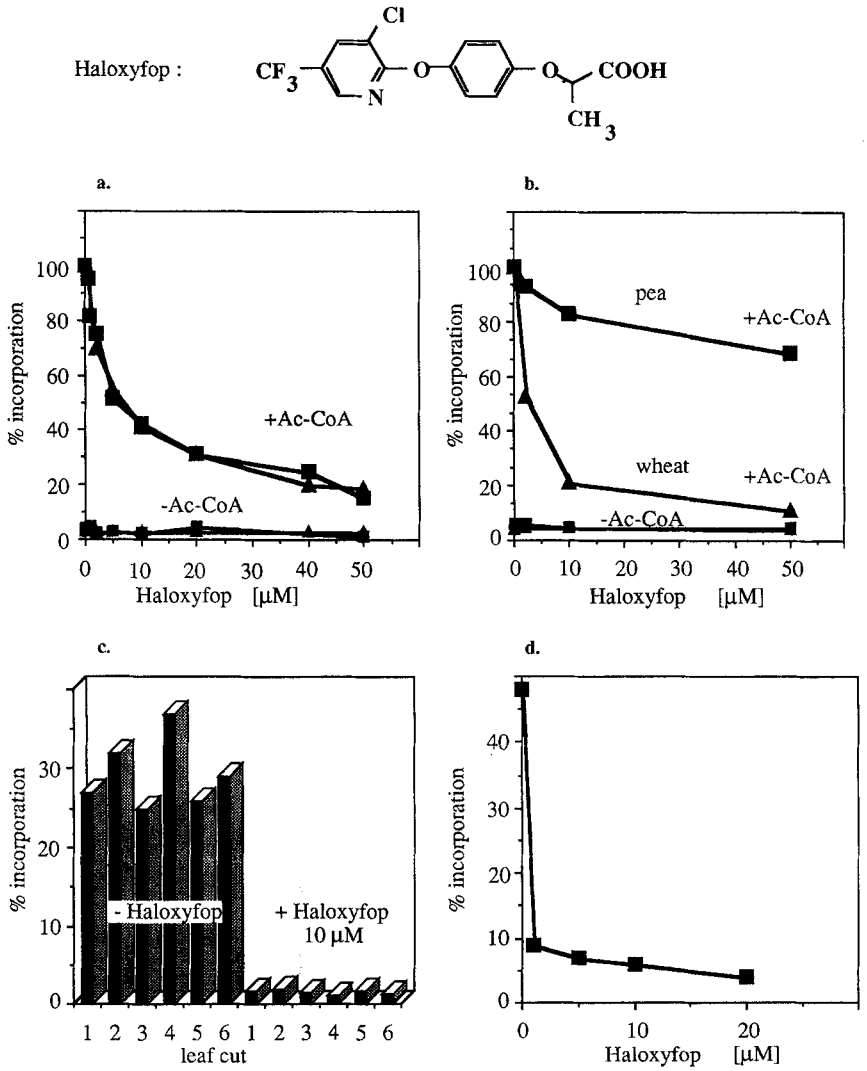


*Fig. 3.* Effect of haloxyfop on wheat ACC activity. a. Incorporation of bicarbonate into malonate by wheat germ ACC (triangles, wheat germ extract partially purified throughout gel filtration on Sephadex G-100 as described in this paper; squares, wheat germ extract purchased from Amersham) at different concentrations of haloxyfop. b. Incorporation of bicarbonate into malonate by ACC in protein from purified, intact wheat (triangles) and pea (squares) chloroplasts at different concentrations of haloxyfop. c. Incorporation of acetate into fatty acids in wheat leaves at 10 $\mu$M haloxyfop. d. Incorporation of acetate into fatty acids in wheat leaves (average values of two independent measurements) at different concentrations of haloxyfop.

of ACC from rat, chicken and yeast [1, 11, 17] (X represents residue not determined, B = D or N):

| | ↓ CNBr |
|---|---|
| Wheat ACC | MXXKGVIKKVVDBE |
| Rat ACC | $MQEKGVINDILDWK_{2208}$ |
| Chicken ACC | $MQEKGAITDILDWK_{2186}$ |
| Yeast ACC | $MVAKGVISKELEWT_{2120}$ |

The wheat ACC cDNA cloned so far [P. Gornicki and R. Haselkorn, unpublished results] does not include sequence coding for this peptide motif, but the amino acid sequence will be helpful in identifying additional cDNA clones encoding the 220 kDa polypeptide.

Several years ago it was shown that aryloxyphenoxypropionates and cyclohexanediones, powerful herbicides effective against monocot weeds, inhibit fatty acid biosynthesis in sensitive plants. Recently it has been determined that ACC is the target enzyme. Dicotyledonous plants are resistant to these compounds, as are other eukaryotes and prokaryotes. The mechanism of inhibition/resistance of the enzyme is not known (see [10] for review). The inhibitory effect of some of these herbicides on the wheat germ enzyme has been reported previously [15].

We have tested the effect of haloxyfop, one of the aryloxyphenoxypropionate herbicides, on the activity of ACC from wheat germ and from wheat seedling leaves (Fig. 3). For the *in vitro* assay of ACC activity, 1–8 $\mu$l aliquots of ACC preparations were incubated for 45 min at 37 °C with 20 $\mu$l of 100–200 mM KCl, 200 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 2mM $^{14}C$-$NaHCO_3$, and where indicated 1 mM Ac-CoA, in a final volume of 40 $\mu$l. The reaction was stopped by adding 4 $\mu$l of concentrated HCl 30–40 $\mu$l aliquots of the reaction mixture were spotted on filter paper and dried, and acid-stable radioactivity was measured using scintillation cocktail. Haloxyfop was added as the Tris salt of the acid, generously supplied by J.Secor of Dow-Elanco.

For the *in vivo* assay of ACC activity, 2-week old seedlings of wheat (*Triticum aestivum* cv. Era) were cut about 1 cm below the first leaf and transferred to a 1.5 ml micro tube containing $^{14}C$-sodium acetate and haloxyfop (Tris salt) for 4–6 h. The leaves were then cut into small pieces and treated with 0.5 ml of 40% KOH for 1 h at 70 °C, and then with 0.3 ml of $H_2SO_4$ and 20 $\mu$l of 30% TCA on ice. Fatty acids were extracted with three 0.5 ml aliquots of petroleum ether. The organic phase was washed with 1 ml of water. Incorporation of $^{14}C$-acetate into fatty acids is expressed as the percentage of the total radioactivity taken up by the seedlings, present in the organic phase.

As expected, the enzyme from wheat germ or from wheat chloroplasts was sensitive to the herbicide at very low levels (Fig. 3). 50% inhibition occurs at about 5 and 2 $\mu$M haloxyfop, respectively. For comparison, the enzyme from pea chloroplasts is relatively resistant (50% inhibition occurs at $>50$ $\mu$M haloxyfop). Finally, the *in vivo* incorporation of $^{14}C$-acetate into fatty acids in freshly cut wheat seedling leaves is even more sensitive to the herbicide (50% inhibition occurs at $<1$ $\mu$M haloxyfop) and this seems to provide a convenient assay for both ACC and haloxyfop.

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## References

1. Al-feel W, Chirala SS, Wakil SJ: Cloning of the yeast FAS3 gene and primary structure of yeast acetyl-CoA carboxylase. Proc Natl Acad Sci USA 89: 4534–4538 (1992).
2. Alix J-H: A rapid procedure for cloning genes from $\lambda$ libraries by complementation of *E. coli* defective mutants: application to the *fabE* region of the *E. coli* chromosome. DNA 8: 779–789 (1989).
3. Egin-Buhler B, Ebel J: Improved purification and further characterization of ACC from culture cells of parsley. Eur J Biochem 133: 335–339 (1983).

4. Egin-Buhler B, Loyal R, Ebel J: Comparison of acetyl-CoA carboxylase from parsley cell culture and from wheat germ. Arch Biochem Biophys 203: 90–100 (1980).
5. Fernandez MD, Lamppa GK: Acyl carrier protein import into chloroplasts. J Biol Chem 266: 7220–7226 (1991).
6. Harwood JL: Fatty acid metabolism. Annu Rev Plant Physiol Plant Mol Biol 39: 101–138 (1988).
7. Jahnen-Dechent W, Simpson RJ: A method for preparing proteins and peptides for microsequencing. Plant Mol Biol Rep 8: 92–103 (1990).
8. Li S, Cronan JE: The gene encoding the biotin carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase. J Biol Chem 267: 855–863 (1992).
9. Li S, Cronan JE: The genes encoding the two carboxyltransferase subunits of *Escherichia coli* acetyl-CoA carboxylase. J Biol Chem 267: 16841–16847 (1992).
10. Lichtenthaler HK: Mode of action of herbicides affecting acetyl-CoA carboxylase and fatty acid biosynthesis. Z Naturforsch 45c: 521–528 (1990).
11. Lopez-Casillas F, Bai DH, Luo X, Kong IS, Hermodson MA, Kim KH: Structure of the coding sequence and primary amino acid sequence of rat Acetyl-coenzyme A carboxylase. Proc Natl Acad Sci USA 85: 5784–5788 (1988).
12. Luo X, Park K, Lopez-Casillas F, Kim K: Structural features of acetyl-CoA carboxylase gene: mechanism for the generation of mRNAs with 5′-end heterogeneity. Proc Natl Acad Sci USA 86: 4042–4046 (1989).
13. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
14. Muramatsu S, Mizuno T: Nucleotide sequence of the *fabE* gene and flanking regions containing a bent DNA sequence of *Escherichia coli*. Nucl Acids Res 17: 3982 (1989).
15. Rendina AR, Felts JM, Beaudoin JD, Craig-Kennard AC, Look LL, Paraskos SL, Hagenah JA: Kinetic characterization, stereoselectivity and species selectivity of the inhibition of plant acetyl-CoA carboxylase by the aryloxyphenoxypropionic acid grass herbicides. Arch Biochem Biophys 265: 219–225 (1988).
16. Slabas AR, Hellyer A: Rapid purification of a high molecular weight subunit polypeptide form of rape seed acetyl-CoA carboxylase. Plant Sci 39: 177–182 (1985).
17. Takai T, Yokoyama C, Wada K, Tanabe T: Primary structure of chicken liver acetyl-coenzyme A carboxylase deduced from cDNA sequence. J Biol Chem 263: 2651–2657 (1988).
18. Witters LA, Kemp BE: Insulin activation of acetyl-CoA carboxylase by inhibition of the 5′-AMP-activated protein kinase. J Biol Chem 267: 2864–2867 (1992).
19. Wurtele ES, Nikolau BJ: Plants contain multiple biotin enzymes: discovery of 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase in the plant kingdom. Arch Biochem Biophys 278: 179–186 (1990).