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Purification and partial amino-acid sequence of gibberellin 20-oxidase from *Cucurbita maxima* L. endosperm

Theodor Lange

Pflanzenphysiologisches Institut und Botanischer Garten der Universität Göttingen, Untere Karspüle 2, D-37073 Göttingen, Germany; FAX: 49 (551) 397823

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Abstract. Gibberellin (GA) 20-oxidase was purified to apparent homogeneity from *Cucurbita maxima* endosperm by fractionated ammonium-sulphate precipitation, gel-filtration chromatography and anion-exchange and hydrophobic-interaction high-performance liquid chromatography (HPLC). Average purification after the last step was 55-fold with 3.9% of the activity recovered. The purest single fraction was enriched 101-fold with 0.2% overall recovery. Apparent relative molecular mass of the enzyme was 45 kDa, as determined by gel-filtration HPLC and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, indicating that GA 20-oxidase is probably a monomeric enzyme. The purified enzyme degraded on two-dimensional gel electrophoresis, giving two protein spots: a major one corresponding to a molecular mass of 30 kDa and a minor one at 45 kDa. The isoelectric point for both was 5.4. The amino-acid sequences of the amino-terminus of the purified enzyme and of two peptides from a tryptic digest were determined. The purified enzyme catalysed the sequential conversion of [^{14}C]GA_{12} to [^{14}C]GA_{15}, [^{14}C]GA_{24} and [^{14}C]GA_{25}, showing that carbon atom 20 was oxidised to the corresponding alcohol, aldehyde and carboxylic acid in three consecutive reactions. [^{14}C]Gibberellin A_{53} was similarly converted to [^{14}C]GA_{44}, [^{14}C]GA_{19}, [^{14}C]GA_{17} and small amounts of a fourth product, which was preliminarily identified as [^{14}C]GA_{20}, a C_{19}-gibberellin. All GAs except [^{14}C]GA_{20} were identified by combined gas chromatography-mass spectrometry. The cofactor requirements in the absence of dithiothreitol were essentially as in its presence (Lange et. al., Planta **195**, 98–107, 1994), except that ascorbate was essential for enzyme activity and the optimal concentration of catalase was lower.

Abbreviations: BSA = bovine serum albumin; DEAE = diethylaminoethyl; DTT = dithiothreitol; GA_n = gibberellin A_n; GC-MS = combined gas/chromatography-mass spectrometry

Key words: *Cucurbita* (seed, endosperm) – Gibberellin biosynthesis – Gibberellin 20-oxidase (purification) – 2-Oxoglutarate-dependent dioxygenases (plant)

Introduction

Gibberellin (GA) plant hormones are biosynthesised via a complex pathway (Graebe 1987; Lange et. al. 1993a, b). Several of the enzymes involved in the later steps of this pathway have been partially purified from various plant sources and characterised with respect to their cofactor requirements and other general properties (Smith and MacMillan 1984, 1986; Gilmour et al. 1987; Kwak et al. 1988; Lange and Graebe 1989; Smith et al. 1990, 1991; Griggs et al. 1991; Lange et al. 1994). Purification, however, has proved very difficult because GA biosynthetic enzymes are instable and occur in very low concentrations in most plant tissues. Consequently, it has not been possible to prepare proteins sufficiently pure for a full characterisation of their properties or for the production of antibodies, or other probes, to be used for cloning the corresponding cDNA.

Endosperm of immature *Cucurbita maxima* seeds contains higher concentrations of GA biosynthetic enzymes than any other plant material investigated so far (Graebe and Lange 1990; Lange and Graebe 1993). The preceding paper describes the partial purification of three enzymes involved in GA biosynthesis from this tissue and their characterisation as 2-oxoglutarate-requiring dioxygenases (Lange et al. 1994). Of these enzymes, GA 20-oxidase was selected for further purification because it plays a special role in the modification of the GA skeleton. Oxidation at carbon atom 20 results in loss of this atom and the formation of the physiologically relevant C_{19}-GAs (Fig. 1). This paper describes the purification to apparent homogeneity of the 20-oxidase, some characteristics of the purified enzyme and its partial sequencing as a prerequisite for molecular cloning. The pure enzyme could only be obtained in very low yield. Recombinant protein

will be needed for full characterisation of its molecular and catalytical properties.

Materials and methods

Plant material. Plants of *Cucurbita maxima* L. cv. 'Riesenmelone, gelb genetzt' (seeds from van Waveren, Göttingen, Germany) were field-grown in the Botanical garden, University of Göttingen, in the summers of 1987–89. Seeds with cotyledons of 30% of the seed lumen (Graebe 1972) were harvested from several fruits, and the endosperm was collected, immediately frozen and stored in liquid N_2.

Labelled GA-substrates. [1, 7, 12, 18-$^{14}C_4$]GA_{12} ($6.6 \cdot 10^{12}$ Bq·mol^{-1}) and [1, 7, 12, 18-$^{14}C_4$]GA_{53} ($5.4 \cdot 10^{12}$ Bq·mol^{-1}) were prepared from R-[2-^{14}C] mevalonic acid ($1.96 \cdot 10^{12}$ Bq·mol^{-1}, Amersham International, Amersham, UK) by incubations with a cell-free system from *C. maxima* endosperm (Lange and Graebe 1993).

Standard enzyme assay. 20-Oxidase activity was determined by the rate of formation of [^{14}C]GA_{15} from [^{14}C]GA_{12}. Before they were assayed, enzyme preparations were diluted to the concentrations given in the individual experiments with 100 mM Tris-HCl buffer, pH 7.0 (measured at 30° C), containing 2 mg·ml^{-1} bovine serum albumin (BSA). The diluted enzyme preparation (85 µl) was supplemented with 5 µl of a cofactor mixture (4 mM 2-oxoglutarate, 4 mM ascorbate, 0.5 mM $FeSO_4$, 2 mg·ml^{-1} BSA, and 0.1 mg·ml^{-1} catalase, final concentrations) and equilibrated at 30° C for 5 min, after which the reaction was started by adding [^{14}C]GA_{12} (100 pmol) dissolved in 10 µl 100 mM Tris-HCl buffer, pH 7.0 (30° C). The incubation mixtures contained less than 0.02 mM dithiothreitol (DTT), 10 mM NaCl and 0.1% methanol originating from the diluted enzyme preparation. The reaction was stopped after 5 min by adding 10 µl acetic acid, which lowered the pH to about 3.2. Variations in the incubation conditions are given in the figure legends to the individual experiments.

Extraction, separation and identification of reaction products. Incubation products were recovered by solid-phase extraction on C_{18} reverse-phase Sep-Pak cartridges (Waters Ass., Milford, Mass., USA). Practically no radioactivity was lost by this method. The samples were then analysed by C_{18} reverse-phase high-performance liquid chromatography (HPLC) with on-line radio monitoring as described by Lange and Graebe (1993). The loss in the transfer to the HPLC-system was determined to be 13% ± 1% (± SD, $n = 15$). The values in per cent of recovered radioactive products are given as determined by on-line detection, without corrections for any loss. The identity of radio-labelled GA substrates and products was confirmed by combined gas chromatography-mass spectrometry (GC-MS) of methyl esters, trimethylsilyl ethers as described previously (Lange et al. 1993b).

Homogenisation and centrifugation. Enzyme extraction, $(NH_4)_2SO_4$ precipitation and Sephadex G-100 gel-filtration chromatography were accomplished at 4° C. Frozen endosperm (250 g) was pulverised in a Waring blender with 1 vol. (w/v) of 200 mM Tris-HCl, pH 7.9, containing 8 mM DTT, and centrifuged at 20000·*g* for 20 min. For determination of 20-oxidase activity, 1 ml of the supernatant was applied to a gel-filtration PD-10 column (Pharmacia, Freiburg, Germany; containing Sephadex G-25 medium gel) to remove low-molecular-weight compounds. The column was equilibrated and eluted with 100 mM Tris-HCl, pH 7.9, containing 4 mM DTT. The protein fraction was collected and then frozen and stored in liquid N_2. The remaining supernatant was fractionated by $(NH_4)_2SO_4$ precipitation.

Ammonium-sulphate precipitation and Sephadex G-100 gel-filtration chromatography. The protein in the 20000·*g* supernatant which precipitated between 50–70% saturated $(NH_4)_2SO_4$ was collected by centrifugation at 15000·*g* for 15 min and dissolved in 51 ml 100 mM Tris-HCl buffer, pH 7.9, containing 4 mM DTT. One millilitre of the dissolved precipitate was desalted using a PD-10 column as described above. The remaining 50 ml were applied to a Sephadex G-100 column (68 cm long, 7 cm i.d.; Pharmacia). The column was equilibrated and eluted with 100 mM Tris-HCl, pH 7.9, containing 4 mM DTT at a flow rate of 81.6 ml·h^{-1}. Fractions (13.6 ml) were collected and samples (5 µl) were assayed for 20-oxidase activity. The chromatography over Sephadex G-100 was repeated four times with new lots of enzyme preparation. Active fractions from all five runs (eluting between 1197 and 1455 ml) were pooled and concentrated 44-fold by ultrafiltration through an Amicon Diaflo PM-10 membrane (43 mm diameter, exclusion limit 10 kDa) in an Amicon-52 cell (Amicon, Witten, Germany). The concentrated fractions were frozen and stored in liquid N_2.

Diethylaminoethyl (DEAE)-anion-exchange HPLC. Protein HPLC was performed at 24° C and a flow rate of 1 ml·min^{-1} using a Waters HPLC system (Waters 650 Advanced Purification System; Waters). The enzyme preparation after chromatography on Sephadex G-100 (3.8 ml) was diluted with double-distilled H_2O (12 ml), containing 4 mM DTT, and applied to a DEAE-5PW-silica HPLC column (7.5 cm long, 8 mm i.d., 10 µm; Waters). The column was equilibrated and eluted with 25 mM Tris-HCl, pH 7.2, containing 4 mM DTT and the eluate was monitored with a UV-detector at 280 nm (Lambda-Max 481; Waters). After elution of the excluded material for 25 min, a 60-min linear gradient of 0 to 62.5 mM NaCl in 25 mM Tris-HCl, pH 7.2, containing 4 mM DTT was applied, followed by a 2-min linear gradient to 1 M NaCl in 50 mM Tris-HCl, pH 7.2, containing 4 mM DTT, which was maintained for 6 min. Fractions (1 ml) were collected and aliquots of each fraction (0.1 µl) were assayed for 20-oxidase activity as described above. The active fractions (eluted between 60–66 ml) from altogether 6 runs were pooled and concentrated 8.6-fold in an Amicon-52 cell.

Phenyl-hydrophobic-interaction HPLC. The solution containing the pooled and concentrated fractions after anion-exchange HPLC (3.9 ml) was diluted 1:1 (v:v) with 100 mM Tris-HCl, pH 7.2, containing 4 mM DTT and 4 M NaCl, and applied to a phenyl-5PW-silica column (7.5 cm long, 8 mm i.d., 10 µm; Waters), equilibrated and eluted with 100 mM Tris-HCl, pH 7.2, containing 4 mM DTT and 2 M NaCl. After elution of the unbound protein for 14 min, a 10-min linear gradient of 100 mM Tris-HCl, pH 7.2, containing 4 mM DTT and 2 M NaCl, to 100 mM Tris-HCl, pH 7.2, containing 4 mM DTT and 1 M NaCl was applied, followed by a 30-min linear gradient to 100 mM Tris-HCl, pH 7.2, containing 4 mM DTT and a 10-min linear gradient to 25 mM Tris-HCl, pH 7.2, containing 4 mM DTT and 20 vol.% methanol. Fractions (1 ml) were collected and aliquots of each fraction (0.1 µl) were assayed for 20-oxidase activity as described above.

The whole purification procedure was done twice using different lots of *C. maxima* seeds. The fraction eluted from hydrophobic-interaction HPLC between 24–25 ml in the first run (designated fraction 24–25 ml) was used for two-dimensional electrophoresis, N-terminal sequencing and determination of final enzyme purification and recovery. For two-dimensional electrophoresis and for amino-acid sequencing, a sample (800 µl) of this fraction was concentrated 8-fold, and the buffer was exchanged by repeated (6 times) 10-fold dilution with 50 mM phosphate-buffer, pH 7.3, each time concentrating the diluted sample by centrifugal ultrafiltration. Pooled fractions of the second batch, eluted between 23–27 ml in hydrophobic-interaction HPLC, were used for most enzyme characterisation assays, for obtaining tryptic peptides and, together with fractions 46–55 ml, for molecular-weight determination of the native enzyme by gel-filtration HPLC. Both enzyme preparations were concentrated by centrifugal ultrafiltration in Centricon-10 concentrators (Amicon).

Gel-filtration HPLC and molecular-weight estimation. The apparent molecular weight of the GA 20-oxidase was determined by gel-filtration HPLC using two enzyme preparations after hydrophobic-

interaction HPLC. The samples (80 µl), containing 62 µg protein of fractions 23–27 ml and 190 µg protein of fractions 46–55 ml, were analysed separately on a 200SW-silica gel-filtration column (30 cm long, 8 mm i.d., 10 µm; Waters). The column was equilibrated and eluted at a flow rate of 0.5 ml·min^{-1} with 50 mM Tris-HCl, pH 7.2, containing 4 mM DTT, 0.2 mM EDTA and 250 mM NaCl. Fractions of 0.25 ml were collected and aliquots of each fraction (0.33 µl of the first and 0.63 µl of the second run) were assayed for 20-oxidase activity as described above. The apparent molecular mass was determined using BSA (68 kDa), ovalbumin (45 kDa), myoglobin (17.8 kDa) and cytochrome *c* (12.3 kDa; all from Serva, Heidelberg, Germany) as molecular-weight standards.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples (7.5 µl) of fractions obtained after hydrophobic-interaction HPLC were applied to 10% (w/v) polyacrylamide minigels (8 cm long, 8 cm wide, 0.1 cm thick; Biometra, Göttingen, Germany) according to Laemmli (1970). Proteins were visualised by silver staining according to Heukeshoven and Dernick (1985). Molecular mass was determined, using phosphorylase B (92.5 kDa), BSA (68 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa; all from Serva) as molecular-weight markers (2 µg per lane).

Two-dimensional electrophoresis and isoelectric-point (pI) estimation. Fraction 24–25 ml (0.8 µg protein) after hydrophobic-interaction HPLC was analysed by ultrathin-layer isoeletric focusing in Servalyt Precotes, pH 3.0–10.5 (Serva) according to the manufacturer's instructions. A narrow strip of the gel, containing the protein lane, was applied onto the top of a 10% (w/v) polyacrylamide separation-gel (14 cm long, 10 cm wide, 0.1 cm thick). The remaining space was filled with 3% (w/v) polyacrylamide stacking-gel. The run was performed and the gel was stained with silver as described above for SDS-PAGE. The isoelectric point was determined by comparison of the location of the enzyme with the following markers (4 µg per lane): amyloglucosidase (pI 3.5), ferritin (pI 4.4), BSA (pI 4.7), β-lactoglobulin (pI 5.34), conalbumin (pI 5.9), horse myoglobin (pI 7.3), whale myoglobin (pI 8.3), ribonuclease (pI 9.45) and cytochrome *c* (pI 10.5; all from Serva).

Tryptic digestion and peptide separation. Tryptic peptides were obtained by digesting a sample (17.2 µl, 50 µg protein) of fractions 23–27 ml, obtained by hydrophobic-interaction HPLC, with trypsin (2.5 µg; Sigma, München, Germany) in a total volume of 50 µl 100 mM Tris-HCl (pH 8.0,) at 37° C for 10 h. The peptides were separated by C_{18} reverse-phase HPLC using a Waters HPLC system (Waters model 680 programmer and two pumps, models 6000 A and M-45). The digested sample was applied directly to a Novapak C_{18} column (15 cm long, 8 mm i.d., 5 µm; Radial-Pak Liquid Chromatography Cartridge in a RCM 100 radial compression system, Waters), equilibrated and eluted with 0.1% trifluoroacetic acid in water. The eluate was monitored at 214 nm (Lambda-Max 481; Waters) and the solvent flow rate was 2 ml·min^{-1}. After elution for 5 min, a 75-min linear gradient of 0–50% acetonitrile in 0.1% aqueous trifluoroacetic acid was run, followed by a 5-min linear gradient to 100% acetonitrile in 0.1% trifluoroacetic acid, which was maintained for 10 min. Single peaks were collected, taken to dryness in a Speed-Vac vacuum concentrator (Savant Instrument, Farmingdale, N.Y., USA) and stored at 4° C until sequencing.

Amino-acid sequence determination. Two of the purified tryptic peptides and a sample (40 µg protein) of the undigested fraction 24–25 ml obtained after hydrophobic-interaction HPLC were submitted to the Department of Biochemistry (II) of the Medical Faculty, University of Göttingen, Germany, for amino-acid sequence analysis with an Applied Biosystems 477A Protein Sequencer (Hunkapiller 1988).

Protein determination. Protein was determined according to Sedmak and Grossberg (1977), using BSA as the standard.

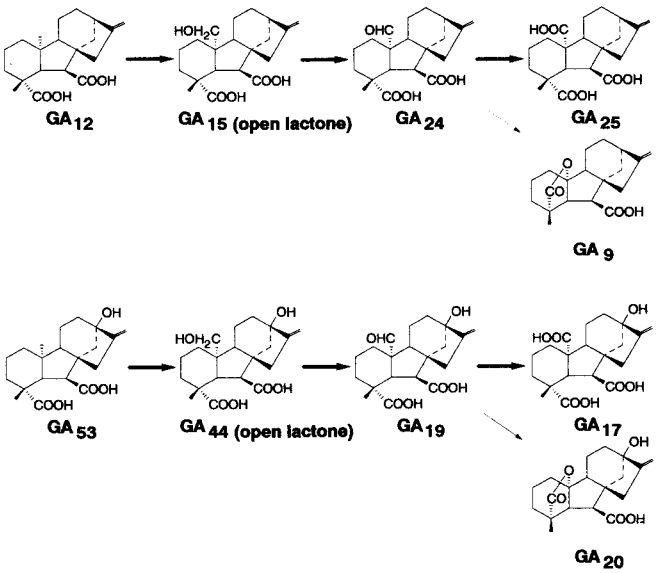

Fig 1. Oxidation of carbon atom 20 in parallel pathways of gibberellin biosynthesis. The 20-methyl groups of GA_{12} and GA_{53} are oxidised via the alcohols and aldehydes to either carboxylic acids (GA_{25} and GA_{17}, respectively), or, after loss of carbon-20, to C_{19}-GAs (GA_9 and GA_{20}). *Thick arrows* indicate preferred reactions in *C. maxima* seeds (Lange et. al. 1993a, b); GA_9 has not been found in this material. In other plant material the production of GA_9 and GA_{20} is often more prominent than that of GA_{25} and GA_{17} (see Graebe 1987)

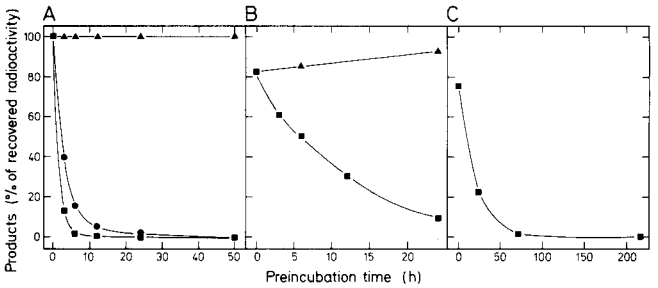

Fig. 2A–C. Influence of acid pH (**A**), cofactors (**B**) and $(NH_4)_2SO_4$ (**C**) on GA 20-oxidase activity from *C. maxima* endosperm during preincubation. **A** Enzyme preparation after Sephadex G-100 gel filtration was diluted 100-fold with 10 mM Bis-Tris-HCl, pH 5.5, containing no additives (■–■; final pH 5.9) or 4 mM DTT (●–●; final pH 5.8), or it was diluted with 10 mM Bis-Tris-HCl, pH 6.5 (▲–▲; final pH 6.9). After preincubation for the times indicated at 24° C, samples (2.8 µg protein) were analysed by standard assay, except that $FeSO_4$ was omitted to avoid interaction with DTT. **B** Gel-filtered enzyme preparation (pH 7.2) was preincubated at 24° C with (■–■) and without (▲–▲) cofactors (4 mM ascorbate, 4 mM 2-oxoglutarate, 0.5 mM $FeSO_4$, 6 mg·ml^{-1} BSA, 0.1 mg · ml^{-1} catalase), and samples (0.69 µg protein) were analysed by standard assay, except for BSA (6 mg·ml^{-1}). **C** Dissolved enzyme preparation after $(NH_4)_2SO_4$ precipitation (pH 7.8), containing 7% $(NH_4)_2SO_4$, was preincubated at 4° C and samples (2.5 µg protein) were analysed by standard assay, except for BSA (6 mg·ml^{-1})

Results

Preliminary experiments. The stability of GA 20-oxidase in the preparation obtained after Sephadex G-100 chromatography was investigated under conditions relevant for the subsequent purification by protein HPLC. En-

zyme activity of the unprotected preparation was reduced 30% by freezing in liquid N_2 and thawing at 24° C but was fully preserved in the presence of DTT (4 mM), even after five cycles of freezing and thawing (data not shown). The diluted Sephadex G-100 preparation (pH 6.9 at 24° C) retained full activity after preincubation for 50 h at 24° C without any additives (Fig. 2A). The presence of DTT (up to 16 mM), methanol (up to 20%), NaCl (up to 500 mM) or pH values of 7.7 and 8.5 did not decrease this stability during at least 24 h (data identical to that of the control). However, preincubation at pH 5.9 caused more than 95% loss of activity after 6 h, and adding DTT did not prevent this inactivation (Fig. 2A). Also the presence of cofactors (at 24° C) or $(NH_4)_2SO_4$ (at 4° C) caused massive loss of activity in relatively short time (Fig. 2B, C).

Since DTT preserved enzyme activity at least during freezing and thawing, it was added to all buffers during purification. However, the presence of DTT during incubation was not desirable, since it is inhibitory under certain circumstances (Lange et al. 1994) and also may lead to artefact production as the protein concentration becomes lower with increasing purity (Graebe and Lange 1990). The samples to be assayed were therefore diluted with buffer without DTT. The cofactor requirements of 20-oxidase, which had originally been optimised in the presence of 4 mM DTT (Lange et al. 1994), were investigated without DTT, using the preparation obtained after anion-exchange HPLC. The Michaelis constants for GA_{12} and 2-oxoglutarate and the dependence on Fe^{2+} remained the same as before (Lange et al. 1994). However, ascorbate, which was merely stimulating in the previous work, was now required for activity (Fig. 3A). The optimal concentration for BSA was 2 $mg \cdot ml^{-1}$ (Fig. 3B) and that for catalase 0.1 $mg \cdot ml^{-1}$ (Fig. 3C), one-tenth of the optimal concentration in the presence of DTT.

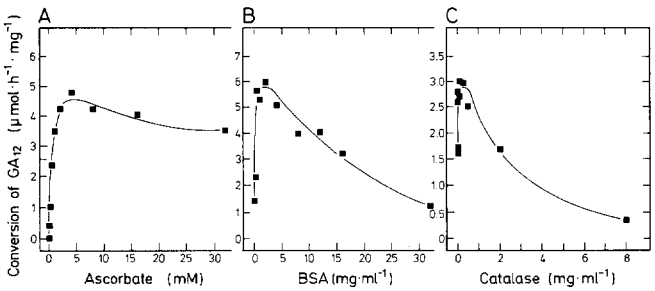

Fig. 3A–C. Concentration curves for ascorbate (**A**), BSA (**B**) and catalase (**C**) Purified enzyme preparation after anion-exchange HPLC (1.2 $\mu g \cdot ml^{-1}$) was incubated as described in *Materials and methods*, except that cofactors were varied as shown

Enzyme purification. The method used by Lange et al. (1994) to purify GA 20-oxidase and other GA oxidases from *C. maxima* endosperm was extended by the introduction of anion-exchange HPLC on DEAE-silica, followed by hydrophobic-interaction HPLC on phenyl-silica. In addition to including DTT in the homogenisation buffer and during all enzyme purification steps, pH was maintained above 7.0 on the basis of the results obtained in the preliminary experiments. Large amounts of protein were applied during column chromatography and the eluted fractions were immediately concentrated in order to reduce loss of enzyme activity due to dilution (Graebe and Lange 1990). The first purification steps, including centrifugation, $(NH_4)_2SO_4$ precipitation and gel-filtration chromatography resulted in five-fold purification and 33% recovery of 20-oxidase activity (Table 1).

Anion-exchange HPLC separated 20-oxidase activity from the bulk of protein (Fig. 4), resulting in nine-fold increase in specific activity and 33% recovery of enzyme activity as calculated relatively to the preceding step (Table 1). Other GA-oxidising activities (GA_{12}-aldehyde 7-oxidase, GA_{25} 3β-hydroxylase and GA_{13} 2β-hydroxylase), which contaminate 20-oxidase after gel filtration (Lange et al. 1994), did not bind to the anion-exchange column, thus separating cleanly from 20-oxidase. Gibberellin 12α-hydroxylase activity, known to be present in *C. maxima* endosperm (Lange et al. 1993a), was not detectable in fractions after anion-exchange HPLC (data not shown).

After hydrophobic-interaction HPLC, a discrete peak of 20-oxidase activity was found in fractions eluting between 23–27 ml with a broader peak appearing at 30–50 ml (Fig. 5). The total increase in specific activity of the

Table 1. Purification of GA 20-oxidase from pumpkin endosperm. One litre of endosperm was extracted and purified as described in *Materials and methods*. Standard assays were performed with each single fraction at each purification step, adjusting protein concentrations to ensure similar conversion of the substrates (approx. 500 $pmol \cdot h^{-1}$). Values for peak fractions are summarised for clarity

Purification step	Protein (mg)	Specific activity ($pkat \cdot mg^{-1}$)	Purification factor	Activity recovered (%)
Homogenate	2500	200	1.0	100
20000·g supernatant	2300	270	1.4	130
$(NH_4)_2SO_4$ (50–70%)	430	360	1.8	31
Sephadex G-100 fractions 1197–1455 ml	170	960	4.9	33
DEAE-HPLC fractions 60–66 ml	6.3	8700	44	11
Phenyl-HPLC				
fractions 21–55 ml	1.8	11000	55	3.8
fractions 23–27 ml	0.19	13000	68	0.51
fractions 27–46 ml	1.2	9400	47	2.3
fractions 46–55 ml	0.30	16000	79	0.93
fraction 24–25 ml	0.055	20000	101	0.22

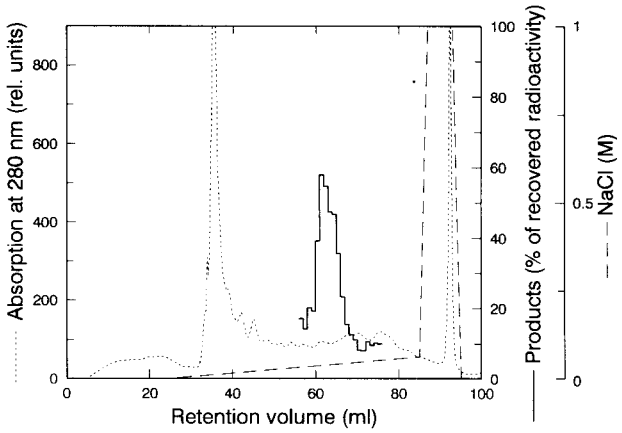

Fig. 4. Purification of GA 20-oxidase by anion-exchange HPLC. Fractions were sampled and assayed as described in *Materials and methods*

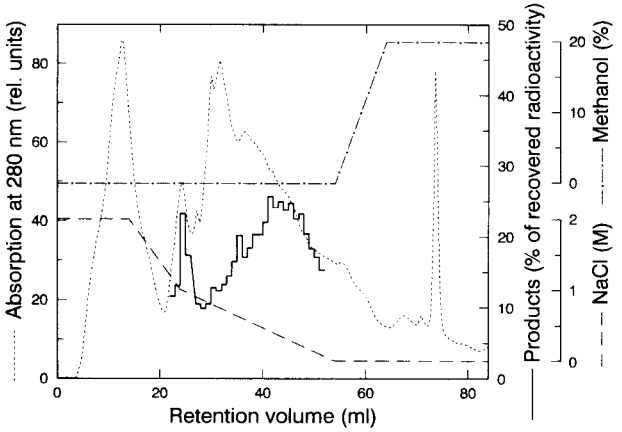

Fig. 5. Purification of GA 20-oxidase by hydrophobic-interaction HPLC. Fractions were sampled and assayed as described in *Materials and methods*

initial peak was 67-fold and 0.5% of enzyme activity was recovered (Table 1). The highest specific activity was contained in the single fraction eluting at 24–25 ml showing 101-fold apparent purification and 0.2% yield. The highest specific activity of the second peak was found in fractions eluting between 46–55 ml with an apparent 79-fold purification and containing 0.9% of the original activity. In all, 3.8% of the total activity was recovered in the two peaks after hydrophobic interaction chromatography, representing 35% of the activity remaining after the previous step (anion-exchange HPLC).

Molecular mass, SDS-PAGE, two-dimensional electrophoresis, and isoelectric point. The apparent molecular mass of 20-oxidase was determined by gel-filtration HPLC on a SW-200 silica column using the two enzyme preparations obtained after hydrophobic-interaction HPLC with the highest specific activity (fractions 23–27 ml and fractions 46–55 ml). Both preparations indicated a molecular mass for GA 20-oxidase of 45 kDa (Fig. 6), confirming the molecular mass of this enzyme previously determined in a partially-purified preparation

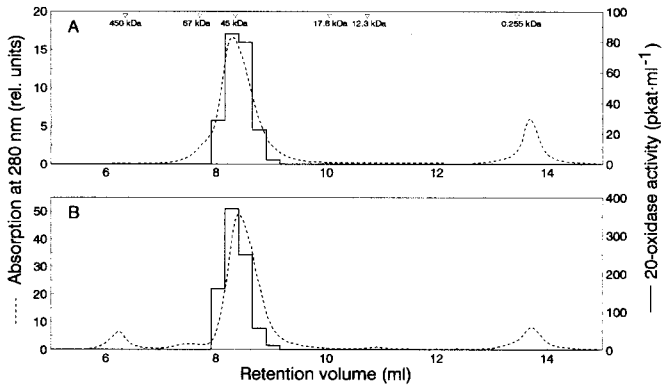

Fig. 6A, B. Analysis of fractions 23–27 ml (**A**) and fractions 46–55 (**B**) ml after hydrophobic-interaction HPLC (see Fig. 5) by gel-filtration HPLC. Fractions were sampled and assayed as described in *Materials and methods*

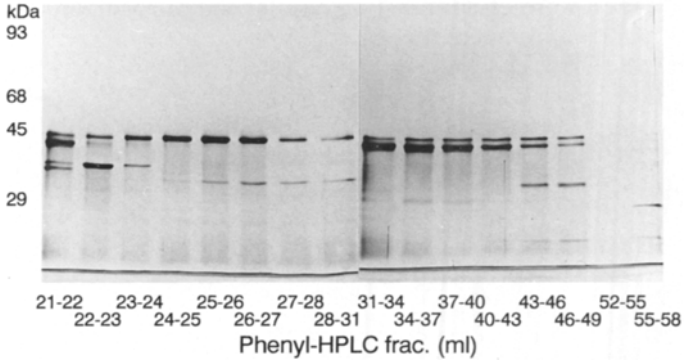

Fig. 7. The SDS-PAGE of fractions with 20-oxidase activity obtained after hydrophobic-interaction HPLC (see Fig. 5)

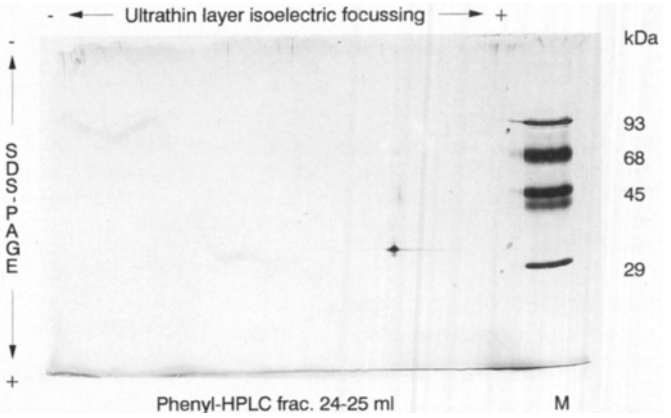

Fig. 8. Two-dimensional electrophoresis of fraction 24–25 ml after hydrophobic-interaction HPLC (see Fig. 5). The molecular-weight markers in lane *M* were separated by SDS-PAGE only

(Lange et al. 1994). The major protein peak in phenyl-HPLC fractions 23–27 ml corresponded to the peak of enzyme activity (Fig. 6A), whereas the major protein peak of phenyl-HPLC fractions 46–55 ml eluted at 42 kDa, slightly displaced from the peak of enzyme activity (Fig. 6B). The peaks appearing in the range of very small molecular masses, close to 14 ml, are probably due to oxidised DTT.

Active fractions after hydrophobic-interaction HPLC were analysed by SDS-PAGE (Fig. 7). One predominant protein band of 44 kDa was found in fractions 23–27 ml with a fainter protein band appearing at 30 kDa. In fraction 24–25 ml the 44-kDa protein band appeared almost pure. It also was present in further fractions, but, from 31 ml on, these fractions also contained a major protein band at 41 kDa.

After two-dimensional electrophoresis of phenyl-HPLC fraction 24–25 ml the most conspicuous protein spot was at 30 kDa with a faint spot appearing at 45 kDa (Fig. 8). The isoelectric point was 5.4 for both spots.

Determination of partial amino-acid sequences. Amino-acid sequencing was accomplished with the purest enzyme preparation after hydrophobic-interaction HPLC (fraction 24–25 ml), revealing unblocked N-terminus with the sequence:

LNEEMKGEYRPPFGG.

The amino-acid sequences of two tryptic peptides were

I: GEYRPPFGGSDESK

II: NFFEDNDSILR.

Thus nine amino acids of the first peptide were also part of the N-terminal amino acid sequence.

Catalytic properties. The metabolism of [^{14}C]GA_{12} and [^{14}C]GA_{53} by 20-oxidase was investigated with the mixture of fractions eluting at 23–27 ml after hydrophobic-interaction HPLC to get enough protein (Table 1, Fig. 7). Increasing concentrations of the enzyme preparation were incubated with the substrates for a constant time and the products were analysed and quantified by radio-HPLC. This method, which has been used for detailed analysis of the GA pathway in cell-free extracts of *C. maxima* seeds (Lange et al. 1993a, b), leads to successively higher reaction rates and, thus, to the accumulation of ever further products in a sequence. The results are similar to those of a time sequence, but less protein is needed and all samples are incubated equally long.

The 20-methyl group of both substrates was oxidised sequentially to the alcohol, aldehyde and carboxylic acid,

Table 2. Metabolism of [^{14}C]GA_{12} (0.72 μM) by GA 20-oxidase, fractions 23–27 ml, after hydrophobic-interaction HPLC. Standard assay, except for an incubation time of 2 h. Products were separated and quantified by HPLC, their identities were confirmed by GC-MS

Protein concentration (μg·ml^{-1})	Products (% of recovered radioactivity)			
	GA_{12}	GA_{15}	GA_{24}	GA_{25}
0	100	0	0	0
0.02	77	23	0	0
0.041	52	41	7	0
0.082	18	50	29	3
0.16	0	12	36	52
0.33	0	4	4	92

Table 3. Metabolism of [^{14}C]GA_{53} (0.52 μM) by GA 20-oxidase, fractions 23–27 ml, after hydrophobic-interaction HPLC. Standard assay, except for an incubation time of 2 h. Products were separated and quantified by HPLC, their indentities, except for that of GA_{20}, were confirmed by GC-MS

Protein concentration (μg·ml^{-1})	Products (% of recovered radioactivity)				
	GA_{53}	GA_{44}	GA_{19}	GA_{17}	GA_{20}
0	100	0	0	0	0
0.2	69	31	0	0	0
0.82	7	80	11	0	2
3.3	0	35	42	21	2
13	0	8	23	62	7
52	0	0	10	84	6

forming [^{14}C]GA_{15}, [^{14}C]GA_{24} and [^{14}C]GA_{25} from [^{14}C]GA_{12} (Table 2, Fig. 1), and [^{14}C]GA_{44}, [^{14}C]GA_{19} and [^{14}C]GA_{17} from [^{14}C]GA_{53} (Table 3, Fig. 1). The identities of these products were confirmed by GC-MS of the methylated and trimethylsilylated samples. At high protein concentrations, low yields of a fourth product chromatographing like [^{14}C]GA_{20}, a C_{19}-GA, were formed from [^{14}C]GA_{53} (Table 3). The amounts of this component were below the detection limit for GC-MS. Gibberellin A_9, the corresponding C_{19}-GA forming from GA_{12} in many pathways, was not found. The purified 20-oxidase converted [^{14}C]GA_{12} more efficiently than it did [^{14}C]GA_{53}. [^{14}C]Gibberellin A_{12}-aldehyde, [^{14}C]GA_{25} and [^{14}C]GA_{13} were not metabolised by the enzyme preparation, thus showing the absence of other GA-oxidising activities (data not shown).

Discussion

Gibberellin 20-oxidase from *Cucurbita maxima* endosperm was purified 101-fold and to apparent homogeneity, with a total recovery of 0.2% in the purest chromatographic fraction. The purification protocol, recently used to separate three 2-oxoglutarate-dependent dioxygenases involved in GA biosynthesis from the same material (Lange et al. 1994), was extended by anion-exchange and hydrophobic-interaction HPLC. Ion-exchange HPLC was the most efficient purification step, increasing the specific activity by a factor of 9, and the resulting preparation was free of other GA oxidase activities. Hydrophobic-interaction HPLC was the decisive final step giving pure enzyme in one fraction. The elution of 20-oxidase activity over a broad range was a consequence of the flat gradient used. Steeper gradient produced more-distinctive peaks, but no single fraction pure enough to be used for amino-acid sequencing (data not shown).

After hydrophobic-interaction HPLC, the fraction with the highest specific activity gave a single protein band with an apparent molecular mass of 44 kDa on SDS-PAGE (fraction 24–25, Fig. 7). Two other protein bands appeared in the following phenyl-HPLC fractions, a faint one at 30 kDa (starting at 25 ml) and a major one at 41 kDa (starting at 31 ml). However, these proteins are

unlikely to contain 20-oxidase activity, because their maximal intensities on SDS-PAGE do not coincide exactly with the 20-oxidase activity as eluted from the phenyl-HPLC column (Fig. 5). Furthermore, gel-filtration HPLC of the fractions containing 20-oxidase activity (Fig. 6), indicated a single component with molecular mass 45 kDa, confirming the homogeneity of the enzyme. Molecular mass and isoelectric point are similar to those of 20-oxidases from other species (Gilmour et al. 1987; Lange and Graebe 1989).

Purified 20-oxidase is apparently degraded during two-dimensional electrophoresis. Two protein bands appeared, a faint one at 45 kDa, the molecular mass of the intact protein, and a major one at 30 kDa. The cause of this degradation is not fully understood, but since SDS-PAGE did not effect the molecular mass of 20-oxidase, it probably occurred during isoelectric focusing, perhaps as a result of cleavage at the low pH.

Gibberellin 20-oxidase belongs to the class of 2-oxoglutarate-dependent dioxygenases (Lange et al. 1994). Its properties are typical for these enzymes and similar to those of GA oxidases from other sources (Smith and MacMillan 1984, 1986; Lange and Graebe 1989; Smith et al. 1990). In the present work, enzyme activity was stabilised by DTT during freezing and thawing but rapidly lost at acid pH or in the presence of ammonium sulphate or standard cofactors. Accordingly, endogenous cofactors were removed rapidly by ammonium-sulphate precipitation with subsequent gel filtration on Sephadex G-100. Dithiothreitol was included during all purification steps, although this turned out to be important only during freezing and thawing, and the pH was maintained above 7. Whenever possible, protein concentration was kept above 1 $mg \cdot ml^{-1}$, because GA 20-oxidase is extremely unstable on dilution, which becomes a problem as the purity increases (Graebe and Lange 1990). Since BSA is the most effective agent to overcome this difficulty (Graebe and Lange 1990), it was included in all assays. Dithiothreitol stimulates GA 20-oxidase activity, provided catalase is present in relatively high concentration (1 $mg \cdot ml^{-1}$; Lange et al. 1994), but is inhibitory at low catalase concentrations (Lange and Graebe 1989). The optimal concentration of catalase was found to be 10 times lower in the absence of DTT than in its presence. The other cofactor requirements were, therefore, reinvestigated at low catalase concentration and in the absence of DTT. The Michaelis constants for GA_{12} and 2-oxoglutarate remained unchanged under these conditions, but the requirement for ascorbate was absolute, rather than merely stimulating as had been found in the presence of DTT (Lange et al. 1994). Ascorbate is involved in the reaction cycle by reducing FeO^{III} (Hanauske-Abel and Günzler 1982; DeJong and Kemp 1984; Myllylä et al. 1984). Catalase and BSA stimulate the activity of other 2-oxoglutarate-dependent dioxygenases as well (Rhoads et al. 1967; Rhoads and Udenfriend 1970; Smith and MacMillan 1984; Kwak et al. 1988; Lange and Graebe 1989).

Pure 20-oxidase catalysed the oxidation of the 20-methyl groups in GA_{12} and GA_{53} via the alcohols and aldehydes to the carboxylic acids of GA_{25} and GA_{17}, respectively, in three consecutive reactions (Fig. 1). A fourth product, according to preliminary identification GA_{20}, a C_{19}-GA, was formed in low yield from GA_{53}. These sequences, which are known to occur in *C. maxima* seeds (Lange et al. 1993a, b), have also been demonstrated step by step in cell-free systems from *C. maxima* and *Pisum sativum*, showing that the loss of carbon-20 occurs at the oxidation stage of the aldehyde (see Hedden 1983; Graebe 1987). Since, in the present work, 20-oxidase was apparently homogeneous, it is likely that one enzyme catalyses all three or four reactions. The low yield of putative GA_{20} does not in itself suggest that its formation is due to small amounts of a contaminating protein, since a low yield of C_{19}-GAs is typical for cell-free systems from *C. maxima* seeds (Hedden 1983; Lange et al. 1993a, b). Partially-purified 20-oxidases from spinach and pea also catalyse several oxidation steps at carbon atom 20 of the GA molecule, but in these cases the preparations used contained several proteins and it was not clear whether one or more enzymes catalysed the steps (Gilmour et al. 1987; Lange and Graebe 1989). Interestingly, another multifunctional 2-oxoglutarate-dependent dioxygenase, thymine hydroxylase from *Neurospora crassa* and *Rhodotorula glutinis*, also catalyses the oxidation of a methyl group to a carboxylic acid in three successive reactions (Bankel et al. 1977; Warn-Cramer et al. 1983; Thornburg et al. 1993).

The amino-acid sequences of the N-terminus and one of the tryptic peptides from the purified 20-oxidase show an identical sequence of nine amino acids and do not include sequences conserved in other plant dioxygenases (cf. Britsch et al. 1993). A peptide with part of this amino-acid sequence has been synthesised and used to raise polyclonal antibodies. These antibodies detect, specifically, 20-oxidase clones close to full length in a cDNA expression library, derived from *C. maxima* cotyledons (Lange et al. 1994). The amino-acid sequences of the N-terminus and the two tryptic peptides were part of the deduced amino-acid sequence encoded by the cloned and sequenced 20-oxidase cDNA. This confirms that the major protein in fraction 24–25 ml (Fig. 7) is the 20-oxidase.

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