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Purification and partial amino acid sequences of an esterase from tomato

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Abstract

Screening of 18 suspension plant cell cultures of taxonomically distant species revealed that a methyl jasmonate hydrolysing enzyme activity (0.21–5.67 pkat/mg) occurs in all species so far analysed. The methyl jasmonate hydrolysing esterase was purified from cell cultures of *Lycopersicon esculentum* using a five-step procedure including anion-exchange chromatography, gel-filtration and chromatography on hydroxylapatite. The esterase was purified 767-fold to give an almost homogenous protein in a yield of 2.2%. The native enzyme exhibited a M_r of 26 kDa (gel-filtration chromatography), which was similar to the M_r determined by SDS-PAGE and MALDI-TOF analysis (M_r of 28547 kDa). Enzyme kinetics revealed a K_m value of 15 μM and a V_{max} value of 7.97 nkat/mg, an pH optimum of 9.0 and a temperature optimum of 40 °C. The enzyme also efficiently hydrolyzed methyl esters of abscisic acid, indole-3-acetic acid, and fatty acids. In contrast, methyl esters of salicylic acid, benzoic acid and cinnamic acid were only poor substrates for the enzyme. *N*-Methylmaleimide, iodacetamide, bestatin and pepstatin (inhibitors of thiol-, metal- and carboxyproteases, respectively) did not inactivate the enzyme while a serine protease inhibitor, phenylmethylsulfonyl fluoride, at a concentration of 5 mM led to irreversible and complete inhibition of enzyme activity. Proteolysis of the pure enzyme with endoproteinase LysC revealed three peptide fragments with 11–14 amino acids. *N*-Terminal sequencing yielded an additional peptide fragment with 10 amino acids. Sequence alignment of these fragments showed high homologies to certain plant esterases and hydroxynitrile lyases that belong to the α/β hydrolase fold protein superfamily. © 2002 Published by Elsevier Science Ltd.

Keywords: *Lycopersicon esculentum*; Solanaceae; Protein purification; Methyl jasmonate; Jasmonic acid

1. Introduction

In 1962, methyl jasmonate (MeJA) was first identified as an odorant compound in the essential oil of jasmine. During the 80s, jasmonic acid (JA) was discovered as a plant hormone-like substance influencing developmental processes such as growth inhibition, induction of senescence and tuber formation (Parthier, 1991). MeJA and JA were found to occur apparently ubiquitously in the plant kingdom (Hamberg and Gardner, 1992).

In 1990, Farmer and Ryan recognized the essential role of JA as a signal molecule in plant defence (Farmer and Ryan, 1990). When JA or MeJA are applied exogenously to plant cells, accumulation of defence proteins and antimicrobial secondary metabolites (Mueller et al., 1993) is observed. A model was proposed in which receptor activation by the endogenous peptide systemin, a systemic wound signal, or pathogen derived signals leads to liberation of linolenic acid from membrane lipids (Farmer and Ryan, 1992; Ryan, 2000). Linolenate is rapidly converted by constitutive enzymes to JA that directly or indirectly activates defence genes (Mueller, 1997). *Arabidopsis* mutants that are either comprised in their ability to biosynthesise JA or are insensitive to JA are highly susceptible to herbivory and certain pathogens (McConn et al., 1997; Vijayan et al., 1998). Moreover, mutant plants are male sterile and unable to complete their life cycle (Stintzi and Browse, 2000).

Wounding, pathogens and drought have all been shown to transiently induce JA that can rapidly be

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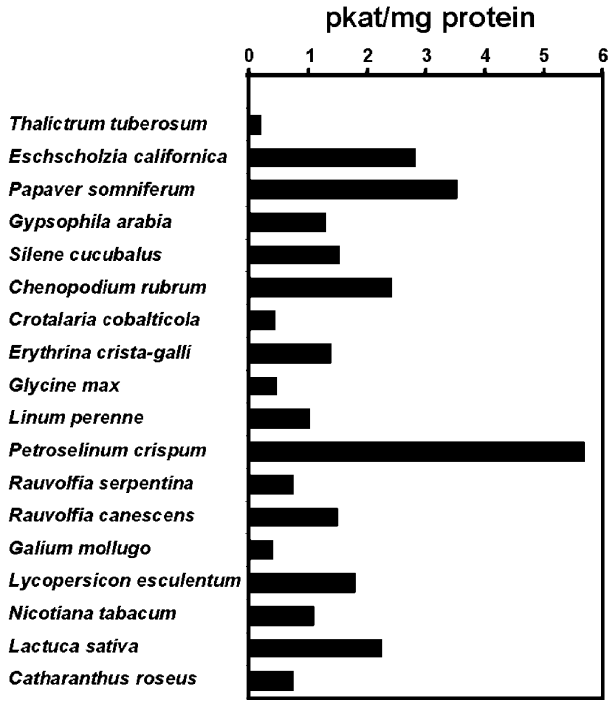

Fig. 1. MeJA cleaving esterase activity in different plant cell cultures. Esterase activity was determined from the soluble protein fraction from different plant cell cultures after removal of low molecular weight contaminants by PD-10 gel-filtration.

methylated to the volatile MeJA (Mueller, 1997). It has been shown that movement of jasmonates among plants may occur via the vapour phase in the form of MeJA (Farmer and Ryan, 1990; Karban et al., 2000; Farmer, 2001). MeJA can readily be taken up by plant cells and may be cleaved by an esterase to regenerate nonvolatile JA within cells. Thus, a volatile long distance signal may be generated by reversible methylation of JA. Alternatively, MeJA may represent itself a signal that needs not to be hydrolyzed to JA to mediate defense gene activation. Since the biological activities of MeJA and JA can not be differentiated in vivo, these two possibilities cannot be distinguished.

Wounding (Creelman et al., 1992) and pathogen attack (Gundlach et al., 1992) often increases levels of JA and MeJA almost simultaneously. Recently, a jasmonic acid carboxyl methyltransferase has been cloned and overexpressed in *Arabidopsis thaliana* (Seo et al., 2001). Transgenic plants had 3-fold elevated levels of endogenous MeJA without altered JA content. Moreover, mutant plants exhibited constitutive expression of jasmonate-responsive genes and showed enhanced level of resistance against the necrotrophic fungus *Botrytis cinera*. Therefore, MeJA formed by jasmonic acid methyltransferase may be an important signal compound in addition to JA.

Characterization of the enzyme catalysing the reverse reaction, MeJA cleaving esterase, would help to clarify the exact role and relative importance of MeJA and JA. Here we report that MeJA cleaving esterase activity is abundant in all plant species so far analysed. The MeJA cleaving enzyme was purified, characterized and partial protein sequence has been obtained by Edman sequencing.

2. Results and discussion

2.1. MeJA esterase activity in plant cell cultures

In total, 18 cell cultures were screened for MeJA esterase activity. Typically, 5 g of plant tissue were shock frozen in liquid nitrogen, grinded, suspended in 10 ml of buffer A and centrifuged (20 min, 12 000 *g*). The supernatant was passed through a PD-10 desalting column and MeJA esterase activity was determined in the crude protein fraction using [methyl-^{3}H]MeJA as substrate. Release of [^{3}H]MeOH was determined after absorption of unreacted MeJA on a RP18-column by scintillography. In some preparations, we also employed methyl [2-^{14}C] jasmonate as substrate to confirm the formation of [2-^{14}C]JA. Both, substrate and product could be identified after TLC with a radioisotope TLC analyser.

MeJA esterase activity was detected in all plant tissues so far analysed and ranged from 0.21–5.67 pkat/mg of protein (Fig. 1). Cultured cells of *Lycopersicon esculentum* were selected as a convenient plant source for enzyme purification, since this cell culture displays excellent growth characteristics yielding kilograms of cell material each week.

2.2. Purification of MeJA cleaving esterase

An outline of the purification of MeJA cleaving esterase from *L. esculentum* is shown in Table 1. For enzyme isolation and purification, 500 g of *L. esculentum* cells were grown and harvested. The crude extract obtained was subjected to $(NH_4)_2SO_4$ fractionation followed by anion-exchange chromatography on Q-Sepharose Fast Flow. At this stage 4.7-fold enzyme enrichment with an overall yield of 48% was obtained. After each chromatographic step, fractions containing MeJA esterase activity were concentrated by ultrafiltration. A further 6-fold enrichment of MeJA cleaving esterase with an overall yield of 30% was achieved when the remaining protein from Q-Sepharose chromatography was subjected to a HiLoad Superdex 200 gel-filtration column. This unusual step at this stage of purification was necessary since a further reduction of contaminating polyphenols could be efficiently accomplished through gel-filtration. Fractions with the highest enzyme activity were combined and used for the next purification step on a hydroxylapatite column. Elution with a linear K-Pi gradient gave a 25-fold purification of the MeJA esterase activity with a yield of 22.4%. Ion exchange chromatography on a Bio-Scale Q2 column

Table 1
Purification of methyl jasmonate cleaving esterase

Purification step	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification (-fold)	Recovery (%)
Crude extract	2503	4430	1.77	1	100
$(NH_4)_2SO_4$ (30–70%)	794	2985	3.76	2.1	67.4
Q-Sepharose fast flow	255	2140	8.39	4.7	48.3
HiLoad Superdex 200	123	1341	10.91	6	30.3
Bio-Scale CHT5	22.5	992.7	44.04	25	22.4
Bio-Scale Q2	2.2	804.3	365.6	207	18.16
Mono Q HR 5/5	0.0723	89.2	1357.1	767	2.2

gave 207-fold enrichment with a specific activity of 365.6 pkat/mg. The remaining enzyme could be further purified on a Mono Q column. During purification, MeJA esterase activity was always found in one single activity peak. In summary, the protein coeluting with MeJA esterase activity was 767-fold purified and showed a specific activity of 1.36 nkat/mg with a 2.2% recovery from the crude protein extract.

In order to obtain sufficient enzyme for partial sequencing, enzyme purification was repeated from 3 kg of *L. esculentum* cells.

2.3. Characterization of MeJA cleaving esterase

A molecular mass of 26 kDa for MeJA cleaving esterase was determined by gel-filtration on a Sephacryl S-100 column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by coomassie or silver staining showed that the fraction purified through the Mono Q column gave two major protein bands with a M_r of ~28 and ~25 kDa (Fig. 2). Careful analysis of the fractions eluting before and after the fraction with maximum MeJA esterase activity revealed that esterase activity corresponded to the stronger protein band with a M_r of ~28 kDa on SDS-PAGE. The accurate mass of this protein determined by ESI-TOF MS was 28547.714 Da (s.d. 237.339 Da). Since the smaller 25 kDa protein was of low abundance we used this fraction for enzyme characterization.

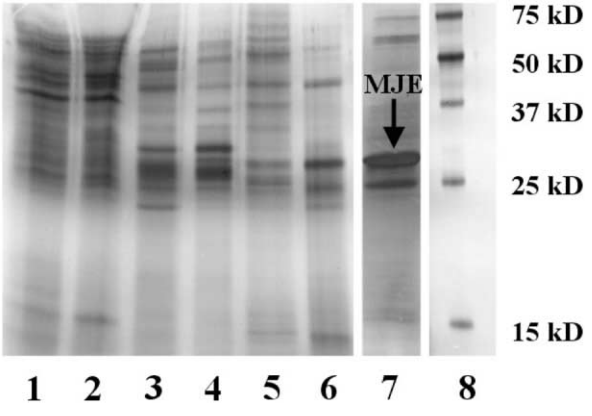

Fig. 2. SDS–PAGE analysis (12.5%, silver staining) of MeJA cleaving esterase (MJE) preparations at different stages of purification. Lane 1: crude extract; lane 2: $(NH_4)_2SO_4$ fraction (30–70% saturation); lane 3: Q-Sepharose fast flow; lane 4: HiLoad Superdex 200; lane 5: Bio-Scale CHT5; lane 6: Bio-Scale Q2; lane 7: Mono Q HR 5/5; lane 8: molecular weight markers. The arrow indicates the protein band corresponding to MeJA cleaving esterase.

Chromatofocussing of MeJA cleaving esterase on a Mono P column gave an isoelectric point (pI) of 4.7 and examination of the pH-dependence of enzyme activity showed that the highest activity was at pH 9.0, whereas 50% of activity was at pH 5.5 and 10.0 (data not shown). Highest activity of the esterase was observed at a temperature of 40 °C. MeJA cleaving esterase was remarkably stable at room temperature and did not loose enzyme activity even after storage under sterile conditions at room temperature for several days.

No inhibition of the esterase was observed using the cysteine protease inhibitors *N*-ethylmaleimide and iodoacetamide at concentrations up to 10 mM. Addition of bestatin (65 μM) and pepstatin (1 μM), inhibitors of metal and acid proteases, respectively, did not inhibit MeJA cleaving esterase. In contrast, addition of 0.5 or 5 mM phenylmethylsulfonyl fluoride, a serine protease inhibitor, yielded 50% and complete inhibition of the esterase, respectively. MeJA cleaving esterase obeys Michaelis–Menten kinetics from which apparent K_m (15.1 ± 1.4 μM) and V_{max} (7.97 ± 0.5 nkat/mg) values could be deduced. The K_m value for MeJA stresses the enzyme's ability to work effectively with the expected low intracellular concentrations of MeJA and is in the same range as the K_m (38 μM) for JA methyltransferase (Seo et al., 2001). A turnover number of 0.23 s^{-1} was calculated for MeJA cleaving esterase. This value is far below the turnover number found for JA methyltransferase (25 s^{-1}). Thus, the K_{cat}/K_m value for the esterase (0.016 μM^{-1} s^{-1}) is substantially lower than for the JA methyltransferase (0.7 μM^{-1} s^{-1}) indicating that the methyltransferase works more efficient than the esterase at low jasmonate concentrations.

Substrate specificity of MeJA cleaving esterase was tested with various ester compounds at a concentration of 0.5 mM under the standard enzyme reaction conditions described in the Experimental section. MeJA

cleaving esterase efficiently hydrolyzed in addition to MeJA, methyl esters of abscisic acid, indole-3-acetic acid, linolenic acid and linoleic acid as well as the chromogenic substrate *p*-nitrophenyl acetate (Table 2). In contrast, methyl esters of salicylic acid, benzoic acid and cinnamic acid were poor substrates. The amide, leucine-*p*-nitroanilide, was not hydrolyzed.

Table 2
Relative hydrolase activity of tomato MeJA cleaving esterase on various esters and amides

Substrates	Relative activity (mean ± S.D.) (%)
(±)Jasmonic acid methyl ester (MeJA)	100.0 ± 1.0
Indole-3-acetic acid methyl ester	95.9 ± 1.7
Linolenic acid methyl ester	71.7 ± 12.6
Acetic acid *p*-nitrophenyl ester	65.3 ± 11.6
(±)Abscisic acid methyl ester	32.4 ± 6.6
Linoleic acid methyl ester	12.4 ± 2.7
Cinnamic acid methyl ester	4.5 ± 0.6
Benzoic acid methyl ester	3.3 ± 2.7
Salicylic acid methyl ester	2.9 ± 0.8
L-Leucine *p*-nitroanilide	0

All substrates were tested at a 0.5 mM concentration. Incubations were terminated by addition of hydrochloric acid. Substrates and products were extracted with diethyl ether and treated with diazoethane. Aliquots were analyzed by GC-positive chemical ionization-MS and methyl esterase activity was calculated from the peak areas of the $[M+H^+]$ ions of the corresponding methyl esters (unreacted substrate) and ethyl esters (product derivative). In the case of the chromogenic substrates (*p*-nitrophenyl ester and *p*-nitroanilid) product formation was determined photometrically. The relative esterase activity with MeJA was set arbitrarily at 100%. Values are the mean ± S.D. from three independent experiments.

Table 3
Sequences of four peptide fragments (PF) of MeJA cleaving esterase

Peptide fragment	Amino acid sequence
PF 1 (N-terminus)	Lys-Gly-Asp-Lys-Asn-His-Phe-Val-Leu-(X)-His
PF 18 a	Val-Val-Thr-Ile-Leu-Arg-Ser-Glu-Gly-His-Lys
PF 18 b	Tyr-Gly-Ser-Val-His-Arg-Val-Tyr-Val-Val-Cys-Asp-Lys
PF 23	Val-Ser-Val-Leu-Asp-Met-Ala-Ala-Ser-Gly-Ile-Asn-Pro-Lys

2.4. Partial amino acid sequence of the esterase

The proteins with an apparent M_r of 25 and 28 kDa obtained from SDS-PAGE were digested with proteinase LysC and the resulting peptides were separated by HPLC and sequenced. Peptides of the 25 kDa protein were identified as fragments of an abundant tomato heat shock protein, HSP70, by database search. The putative esterase (28 kDa) yielded three peptide fragments (PF) from which their peptide sequences could be obtained (Table 3). Sequencing of the *N*-terminus of the ~28 kDa protein band gave a fourth peptide fragment which lacked the first *N*-terminal amino acids, probably due to partial proteolytic degradation. Comparison of the four peptide sequences against protein databases (NCBI and SWISS-PROT) revealed that the esterase is not yet annotated in the databases. However, all four fragments displayed 36–82% sequence identity to different plant esterases such as polyneuridine aldehyde esterase from *Rauvolfia serpentina*, ethylene-induced esterase from *Citrus sinensis* and the pathogen-inducible protein Pir 7b from *Oryza sativa* as well as cyanohydrin lyases (Table 4). Interestingly, all of these proteins belong to the α/β-hydrolase super family, which is characterized by a conserved arrangement of the components of the catalytic triad. The catalytic triad—nucleophile (Ser, Cys or Asp), acid (Asp or Glu) and a histidine—form the basis of the enzymatic properties of this class of enzymes. Despite high structural homology among different members of this protein family, a low degree of sequence conservation is usually found. Enzymes of this family also display different enzymatic activities. Besides esterases and hydroxynitrile lyases, haloalkane dehalogenases, semialdehyde hydrolases,

Table 4
Sequence comparison of MeJA cleaving esterase peptide fragments against SWISS-PROT database using FASTA3

Enzymes	Sequences with homologies to *N*-terminus	Identity (%)	Sequences with homologies to PF 18a	Identity (%)
	KGDK–NHFVLxH		VVTILRSEGHK	
RS	1 MHSAANA**K**QQK**HFVLVH**	60	29 LKPLLESAGHK 39	46
CS	1 MEEVVGMEEK**HFVLVH**	50	28 LKARLVAGGHR 38	27
ME	1 VTA—**HFVLI**H	50	21 LKPALERAGHK 31	36
OS	1 megsssssk**hfilvh**	40	27 VVTMLRSEGHR 37	82
Enzymes	Sequences with homologies to PF 18b	Identity (%)	Sequences with homologies to PF 23	Identity (%)
	VSVLDMAASGINPK		**YGSVHRVYVVCDK**	
RS	40 **VTAVDLSAAGINP**R53	43	203**YGSVKRAYIFC**NE215	54
CS	39 **VTAVDLAASGIN**MK52	64	204**YGSVKRVYLV**CEE216	69
ME	32 **VTALDMAASGIDP**R45	71	194**YGSIKKVYIWTD**Q206	46
OS	38 **VTALDLAASG**VH**PA**51	57	154**YGSVKRV**CLMAME166	46

Enzymes are: RS, polyneuridine aldehyde esterase from *Rauvolfia serpentina* (Q9SE93); CS ethylene-induced esterase from *Citrus sinensis* (Q94G63); ME, (*S*)-Acetone-cyanohydrin lyase from *Manihot esculenta* (P52705); OS, Pir 7b (*Pseudomonas* inducible protein) from *Oryza sativa* (Q942Y9). SWISS-PROT accession numbers are given in brackets; numbers indicate location of peptides in the enzyme. Identical amino acids are bold.

haloalkane peroxidases and epoxide hydrolases have been found within this structural class (Heikinheimo et al., 1999).

The sequence information reported herein can be used to clone the corresponding esterase gene from tomato. In future, transgenic plants may be produced that are either devoid of MeJA esterase activity or overexpress the MeJA cleaving esterase in order to study the function of the enzyme in plant defence signalling.

3. Experimental

3.1. General experimental procedures

JA methyl ester (racemic mixture) was obtained from Serva (Heidelberg, Germany). [Methyl-^{3}H]methyl iodide (sp. act of 85 Ci/mmol) was from Amersham Pharmacia Biotech (Freiburg, Germany). Solid phase extraction columns (1 ml) were from Amchro (Hattersheim, Germany). Aminopropyl- and RP18-solid phase extraction materials and Polygram SIL G/UV$_{254}$ 0.2 mm thin layer chromatography plates were from Macherey-Nagel (Düren, Germany). Scintillation cocktail Ultima Gold was purchased from Packard (Groningen, The Netherlands). For ultrafiltration, Centricon Plus-20 centrifugal filter devices (membrane type PL-10) from Millipore (Eschborn, Germany) were used. Q-Sepharose Fast Flow material, Superdex 200 prep grade material, Mono Q HR 5/5 column, Mono P HR 5/20 column, Sephacryl S-100 HR 26/60 column and PD-10 desalting columns were from Amersham Pharmacia Biotech (Freiburg, Germany). Bio-Scale CHT5 column and Bio-Scale Q2 column were from Bio-Rad Laboratories (Munich, Germany).

The other chemicals used were of highest purity.

3.2. Plant cell material

Cell suspensions cultures of *L. esculentum* were grown in 1 l Erlenmeyer flasks in Linsmaier and Skoog medium (Linsmaier and Skoog, 1965) for 7 days under continuous light (600 lux) on orbital shakers (100 rpm) at 24±2 °C. The cells were harvested by suction filtration, shock frozen with liquid nitrogen, and stored at−20 °C until use.

3.3. Synthesis of [methyl-^{3}H]MeJA and of [2-^{14}C]JA

Racemic jasmonic acid was prepared by alkaline hydrolysis of the methyl ester (Gundlach et al., 1992). For synthesis of [methyl-^{3}H]MeJA, 500 μg of JA was dissolved in 100 μl of EtOAc and incubated with 1 mCi [methyl-^{3}H]methyl iodide (sp. act of 8.5 Ci/mmol) and 1 mg of K_2CO_3 in a tightly locked 0.5 ml vial at 30 °C for 90 min. The mixture was loaded on an aminopropyl-solid phase extraction column (200 mg) and eluted with 1 ml of Et_2O. The flow through and the eluate containing [methyl-^{3}H]MeJA were collected and taken to almost dryness under a gentle stream of nitrogen. Remaining solvent was allowed to evaporate at room temperature without any manipulations, since considerable losses of labelled MeJA may occur during intense drying. This special precautions are necessary since the boiling point of MeJA is only 110 °C.

Labelled MeJa was analysed by thin layer chromatography (hexane–Et_2O–HOAc, 70:30:1) using unlabelled MeJA and JA as references. The absence of unreacted JA was confirmed by staining with iodine vapour. Radiometric analysis revealed that more than 98% of the radioactivity comigrated with methyl jasmonate. The synthesis yielded 19.9% racemic [methyl-^{3}H]MeJA. Racemic [2-^{14}C]JA was synthesized from from 2-(2*Z*-pentenyl)-2-cyclopenten-1-one and diethyl [2-^{14}C]malonate followed by saponification and decarboxylation of the Michael product in one step as described (Knöfel and Gross, 1988). [2-^{14}C]MeJA was prepared from labelled JA by incubation with diazomethane in diethyl ether.

3.4. Enzyme assays

Standard enzyme incubation mixtures (total volume 30 μl) contained enzyme, 167 μM of [methyl-^{3}H]MeJA (sp. act of 9.09 μCi/mmol) in 50 mM K-Pi buffer at pH 7.5. Incubations were performed at 37 °C for the times indicated (5–60 min). Reactions were terminated by addition of 20 μl of HOAc and 100 μl of H_2O–MeOH (70:30). The mixture was loaded on a RP18-solid phase extraction column (50 mg) equilibrated with H_2O–MeOH (90:10). [^{3}H]MeOH released from the enzyme was eluted with 900 μl of H_2O–MeOH (90:10) and radioactivity was quantified by scintillation counting after addition of 2 ml of scintillation cocktail. Internal standards were used to correct for quenching by MeOH.

Substrate specificity was measured by incubating the purified enzyme with various unlabelled methyl esters at a concentration of 500 μM in a total volumne of 50 μl for 30 min. After an incubation time of 30 min, 10 μl of 1 M hydrochloric acid and 500 μl of diethyl ether were added. The mixture was mixed and centrifuged. The organic layer containing the substrate (methyl ester) and product (free acid) was separated and treated with an etheral solution of diazoethane. The solution was evaporated to 50 μl and one μl was analyzed by GC–MS in the positive chemical ionization mode as described (Gundlach et al., 1992). Product formation was calculated from the peak areas of the $[M+H]^+$ ions of the methyl ester (substrate) and the ethyl ester (representing the derivative of the free acid). In the case of chromogenic substrates, product formation was measured photometrically. The relative activity of MeJA cleaving esterase with MeJA (50% substrate consumption in 30 min) was set arbitrarily to 100%.

In some assays, JA formation from MeJA was confirmed by using Me[2-^{14}C]JA as substrate. Formation of [2-^{14}C]JA by the esterase was detected with a radioisotope TLC analyser. The standard assay solution (total volume 200 μl) consisted of 50 mM K-Pi buffer, pH 7.5, and 50 μM [2-^{14}C]MeJA (sp. act of 0.77 μCi/μmol). After an incubation time of 30 min at 37 C, the reaction was terminated by adding 20 μl HOAc and 400 μl EtOAc. The mixture was mixed and centrifuged. The organic phase was separated and carefully concentrated under a gentle stream of nitrogen. Since MeJA evaporates after prolonged time under a nitrogen stream, the organic phase was not completely evaporated. The concentrate was analysed by a radioisotope TLC analyser on silica plates using hexane–EtOAc–HOAc (70:30:1) as solvent.

3.5. Protein determination

Protein concentration was determined by the method of Bradford using bovine serum albumin (Bradford, 1976).

3.6. Composition of buffers

Buffer A: 50 mM K-Pi (pH 7.5), 20 mM β-mercaptoethanol (EtSH), 10 mM ascorbic acid and 1.5% PVPP; Buffer B: 50 mM K-Pi (pH 7.5), 20 mM EtSH; Buffer C: buffer B containing 1 M KCl; Buffer D: buffer B containing 0.15 M NaCl; Buffer E: 20 mM K-Pi (pH 6.8), 20 mM EtSH, 0.3 mM $CaCl_2$; Buffer F: 500 mM K-Pi (pH 6.8), 20 mM EtSH. Buffer G: 50 mM tricine buffer (pH 8), 20 mM EtSH; Buffer H: buffer G containing 1 M KCl; Buffer I: 50 mM K-Pi (pH 5.5), 20 mM EtSH; Buffer J: buffer I containing 1 M KCl; Buffer K: 25 mM bis–Tris set to pH 6.3 with HCl; Buffer L: 10% polybuffer 74 (Amersham Pharmacia Biotech, Freiburg) at pH 4.0 adjusted with HCl.

3.7. Purification of MeJA cleaving esterase

All operations were performed at 4 °C. Desalting, concentration of the enzyme solution and buffer exchange between all chromatographic steps was performed by ultrafiltration using Centricon Plus-20 centrifugal filter devices.

3.8. Preparation of crude protein extracts

Cells of *L. esculentum* (500 g, fr. wt) were shock frozen with liquid nitrogen, added to 1 l of buffer A and homogenised with a Waring blender operated at maximum speed for 5 min. The homogenate was filtered through cheesecloth and clarified by centrifugation (20 min, 12 000 *g*). The supernatant (1500 ml of crude extract) was used immediately for the next purification step.

3.9. Fractional precipitation with $(NH_4)_2SO_4$

The protein in the resulting supernatant was subjected to $(NH_4)_2SO_4$ precipitation. The protein fraction precipitating between 30–70% saturation was collected after centrifugation (20 min, 12 000 *g*), redissolved in a minimal volume of buffer B and desalted by passage through PD-10 desalting columns equilibrated previously with buffer B yielding the soluble protein fraction (90 ml, 8.8 mg/ml).

3.10. Anion-exchange chromatography on Q-Sepharose fast flow

The soluble protein fraction was loaded on a Q-Sepharose fast flow anion-exchange column (2.6×6 cm) previously equilibrated with buffer B. The column was washed with 250 ml of buffer B and MeJA cleaving esterase activity was eluted with a 50 ml linear KCl-gradient prepared from buffers B and C at a flow rate of 4 ml/min. Fractions containing MeJA cleaving esterase activity (>3 pkat/mg) were combined (24 ml), desalted and concentrated to 3 ml by ultrafiltration.

3.11. Gel-filtration on HiLoad 16/60 Superdex 200 prep grade

For gel-filtration, the above enzyme solution was chromatographed on a 1.6×60 cm column containing Superdex 200 prep grade material equilibrated with buffer D. The MeJA cleaving esterase was eluted with buffer D at a flow rate of 1 ml/min and fractions containing esterase activity (>4 pkat/mg) were combined (35 ml). Sample concentration (1 ml) and buffer exchange to buffer E were accomplished by ultrafiltration.

3.12. Bio-Scale CHT5 ceramic hydroxyapatite chromatography

The Bio-Scale CHT5 column (1.0×6.4 cm) was equilibrated with buffer E. Active protein fractions from gel-filtration columns were applied to a Bio-Scale CHT5 column with a flow rate of 1 ml/min. After washing the column with buffer E for 12 min, proteins were eluted with a gradient (25 min) prepared from buffer E and F. Fractions exhibiting MeJA cleaving esterase activity (>10 pkat/mg) were combined (7 ml) and concentrated to 1 ml by ultrafiltration. Sample buffer was exchanged to buffer G.

3.13. Bio-Scale Q2 anion-exchange chromatography

The above obtained protein solution was loaded on a Bio-Scale Q2 anion-exchange column (0.7×5.2 cm) equilibrated with buffer G. After washing the column for 8 min at a flow rate of 1 ml/min, proteins were eluted with a linear KCl gradient (0–1.0 M) prepared

from buffer G and H. Fractions (>90 pkat/mg) were combined (7 ml) and concentrated to 1 ml using ultrafiltration. Sample buffer was exchanged to buffer I.

3.14. Mono Q HR 5/5 anion-exchange chromatography

The remaining protein solution from the anion-exchange chromatography was loaded on a Mono Q column (0.5×5 cm) with a flow rate of 1 ml/min. The column was washed with buffer I for 8 min. The enzyme was eluted with a linear KCl-gradient (0–1.0 M) prepared from buffer I and J at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected. Enzyme activity was found in fractions containing 200–300 mM KCl and active fractions (>500 pkat/mg) were combined.

3.15. FPLC-chromatofocussing with Mono P HR 5/20 column

For determination of the isoelectric point (pI), the protein solution was loaded on a Mono P HR 5/20 column (0.5×20 cm). Proteins were fractionated (1.0 ml) with 50 ml buffer L at a flow rate of 0.7 ml/min generating a linear pH gradient from pH 6.3 to 4.0. The enzyme eluted after 30 ml of buffer L at a pH of 4.7.

3.16. Molecular weight determinations by Sephacryl S-100 chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrospray ionization time of flight mass spectrometry (ESI-TOF MS)

The native molecular mass of the enzyme was estimated by gel-filtration with a Sephacryl S-100 HR 26/60 column (2.6×60 cm) equilibrated with buffer D. The column was calibrated with transferrin (80 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease a (13.7 kDa) as standard proteins (Amersham Pharmacia Biotech, Freiburg).

Molecular weight under denaturating conditions was determined by SDS–PAGE (12.5%) analysis.

ESI-TOF MS analyses were performed on an ETTAN LC–MS system from Amersham Pharmacia Biotech (Freiburg, Germany). The protein sample was desalted and concentrated on a Trap-Column from Amersham Pharmacia Biotech (Freiburg, Germany) prior ESI-TOF MS analysis. M^+-Spectra were calculated using the software package MagTrans.

3.17. Characterization of MeJA cleaving esterase

The optimum pH of the esterase was determined with the following buffers (500 mM): citrate buffer (pH 4.0, 4.5, 5.0 and 5.5), K-Pi buffer (pH 5.5, 6.0, 6.5, 7.0 and 7.5), Tris–HCl (pH 8.0, 8.5, 9.0, 9.5 and 10.0), glycine–NaOH (pH 9.0, 9.5, 10.0, 10.5 and 11.0)

To determine the optimal temperature for hydrolysis, standard esterase assays using MeJA as substrat were performed at temperatures between 5 and 70 °C.

Kinetic data were fitted to standard equations using the software package Origin. V_{max} and apparent K_m values were determined from the Lineweaver–Burk plot.

For inhibition studies MeJA cleaving esterase (100 μg/ml) was preincubated with different concentrations of inhibitor at room temperature for 15 min. Phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, iodoacetamide, pepstatin were dissolved in EtOH and bestatin was dissolved in MeOH. All inhibitors were diluted with 50 mM K-Pi buffer (pH 6.5, EtSH-free) to the concentration needed. Esterase activity assay was performed as described under Section 3.4 for 15 min at 37 °C. Residual MeJA cleaving esterase activity was expressed as percentage of a control preincubation without inhibitor.

3.18. Sequencing of MeJA cleaving esterase and peptide sequence alignment

MeJA cleaving esterase enriched protein fraction obtained from Mono Q chromatography was applied to SDS–PAGE (12.5%) and Coomassie stained. The dominating protein band at 28 kDa and a minor protein band at 25 kDa were cut out and destained with 10% HOAc. Gel slices were digested with endoproteinase LysC. The resulting peptides were separated by HPLC on a Supersphere 60 RP select B column (Merck, Darmstadt) and sequenced.

For N-terminal sequencing, the 28 kDa protein band was blotted on a PVDF membrane by the semi dry blotting method and sequenced.

Peptide sequences were used to screen the SWISS-PROT sequence library using the sequence analysis program FASTA3.

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