FEBS 18452 FEBS Letters 407 (1997) 105–110

Partial purification and characterization of a jasmonic acid conjugate cleaving amidohydrolase from the fungus *Botryodiplodia theobromae*

Silvia C. Hertel, Hans-Dieter Knöfel, Robert Kramell, Otto Miersch*

Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Saale, Germany

Received 10 February 1997

Abstract A protein preparation from the mycelium of the tropical pathogenic fungus *Botryodiplodia theobromae* revealed a novel peptidase activity. This enzyme was capable of cleaving conjugates of jasmonic acid with α-amino acids. The protein was enriched 108-fold by gel filtration, ion exchange and hydrophobic interaction chromatography. The enzyme was found to be a glycoprotein with a molecular mass of about 107 kDa. The amidohydrolase seems to be very specific with regard to (−)-jasmonic acid and α-amino acids with (*S*)-configuration.

Key words: Jasmonic acid conjugate; Amidohydrolase; Partial purification; Substrate specificity; *Botryodiplodia theobromae*

1. Introduction

Jasmonic acid (JA) and its methyl ester have been shown to occur ubiquitously not only in higher plants [1] but also in several fungi [2,3]. Jasmonates have hormone-like properties and are involved in various events of plant growth and development, e.g. promoting, inducing or inhibiting responses of the plants at the cellular or molecular level [4,5].

In addition to its methyl ester, native JA conjugates with amino acids like tyrosine, tryptophan, and phenylalanine have been reported to occur in flowers of the broad bean plant [6]. JA conjugates with isoleucine, leucine and valine were isolated as endogenous compounds from fruits and young leaves of *Vicia faba* L. [7]. The isoleucine derivative of (−)-jasmonic acid was also reported in pollen of *Pinus mugo* [8]. In addition, conjugates of JA and 9,10-dihydro-JA with amino acids could be isolated from *Gibberella fujikuroi* demonstrating that fungi are able to synthesize this type of conjugates too [9,10].

The stress-mediated conjugation of (−)-JA with amino acids in barley after treatment of leaf segments with several osmolytes, such as sorbitol, mannitol or polyethylene glycol 6000, has been demonstrated [11]. Furthermore, jasmonic acid as well as its conjugate with isoleucine were found to be active inducers of specific abundant proteins, the so-called jasmonate-inducible proteins (JIPs), in barley leaf tissue [12]. These observations raise the question whether the JA conjugate is active per se or alternatively needs cleavage of the amide structure giving free JA prior to the molecular response.

But attempts to hydrolyze JA-amino acid conjugates using commercially available proteases failed (Knöfel, unpublished results).

However, preliminary experiments with the fungus *Botryodiplodia theobromae* using radioactively labelled jasmonic acid isoleucine conjugate showed that this fungus might be able to cleave the conjugate (Miersch, unpublished results). Earlier investigations of *Botryodiplodia theobromae* had indicated that this fungus produced both (+)-7-iso-jasmonic acid and several related structures [13,14], but the occurrence of peptide-like conjugates of JA bound to amino acids in the culture medium or the mycelium had never been shown. These underlined the hypothesis of the putative existence of an amidohydrolytic activity in the fungus, schematically illustrated in Fig. 1.

We now report on the partial purification and characterization of an enzyme from the mycelium of *Botryodiplodia theobromae* cleaving JA-amino acid conjugates.

2. Materials and methods

2.1. Chemicals and substrates

The standard substrates were synthesized by first preparing a mixed anhydride between racemic JA and isobutyl chloroformate in the presence of triethylamine according to [15]. The reaction of this mixed anhydride with the respective (*S*)-amino acid, (*R*)-amino acids, β-Ala, ε-amino caproic acid, amines (*p*-nitroaniline, tyramine) or the peptide (*S*)-Val-(*S*)-Leu resulted in the formation of jasmonoyl conjugates. Mostly, formed diastereomers were separated using column chromatography, and the products were characterized by RP-HPLC [16]. Methylesters (−)-JA-(*S*)-IleOMe and (−)-JA-(*S*)-AlaOMe were prepared from the acids by reaction with ethereal diazomethane for 15 min. Esters (−)-JA-(*S*)-TyrOEt and (−)-JA-(*S*)-IleOBu were prepared with ethanol and *n*-butanol, respectively, using *p*-toluenesulfonic acid as catalyst. Reaction chemicals were from Merck (Darmstadt, Germany) and Sigma (Deisenhofen, Germany). Coronatine and coronafacic acid (Fig. 2) were a generous gift of Dr. F. Greulich (Friedrich-Schiller-University Jena, Germany). Malt extract agar was purchased from Serva (Heidelberg, Germany). All other chemicals were of analytical grade.

2.2. Organism and culture condition

The fungus *Botryodiplodia theobromae* strain D 7/2 (1) isolated from Cuban oranges was maintained and pre-cultured on malt extract agar plates at room temperature in the dark. After 5 days, the mycelium was homogenized with distilled water and used for inoculation of the main culture. The fungus was grown under surface culture conditions at 25–27°C for 7 days in Phytacon pots (Sigma) containing 100 ml of the medium described by Miersch et al. [3]. After harvesting, the mycelium was lyophilized and stored dry at 4°C.

2.3. Enzyme assay

The assay mixture contained 20 nmol (−)-JA-(*S*)-Ile in the standard assay or 20 nmol of another substrate for measuring substrate specificity, 10–100 μg protein and buffer of the corresponding purification step to a total volume of 100 μl. Incubation was carried out at 25°C for 30 min. The hydrolysis was stopped with 5 μl of 5 N HCl. After extraction with 100 μl $CHCl_3$, free (−)-JA was separated from the substrate by chromatography on silica gel plates (Merck), solvent

*Corresponding author.
E-mail: omiersch@ipb.Uni-halle.de

Abbreviations: JA, jasmonic acid; (−)-JA-(*S*)-Ile, *N*-[(−)-jasmonoyl-(*S*)-isoleucine; OBu, *n*-butyl ester; OEt, ethyl ester; OMe, methyl ester

PII S0014-5793(97)00307-4

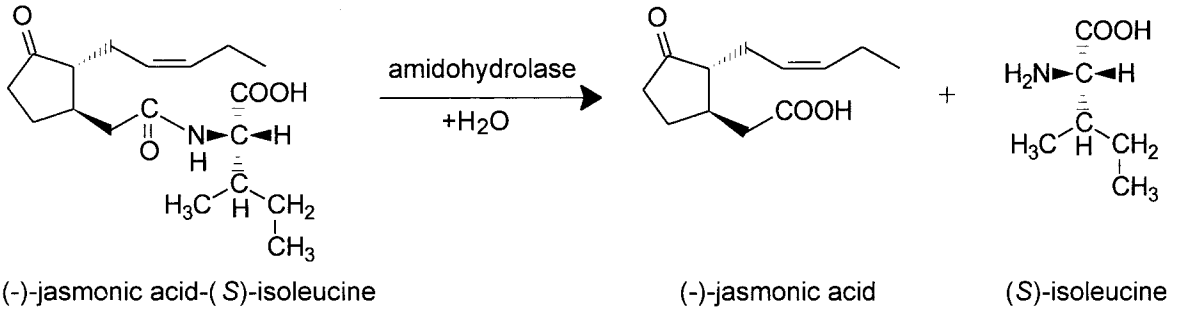

Fig. 1. Scheme of the enzyme reaction of the amidohydrolase

system: $CHCl_3$-EtOAc-HOAc (14:6:1). JA corresponding gel areas were scrapped off, substances recovered with $CHCl_3$ and methylated with ethereal diazomethane for 15 min and evaporated. Quantification was done with an enzyme-linked immunosorbent assay using monoclonal antibodies against (−)-JA methyl ester [17].

In the case of (+)-JA and coronafacic acid (Fig. 2) (corresponding to authentic reference compounds) silica plates were detected by anisaldehyde reagent [18] and spots estimated semi-quantitatively against test spots in the range of 0.1–2 μg.

2.4. Enzyme purification

Buffer systems used were: buffer A (100 mM Tris-HCl, pH 7.4), buffer B (20 mM Tris-HCl, pH 8.2), buffer C (20 mM Tris-HCl, pH 8.2, containing 1 M $(NH_4)_2SO_4$), buffer D (100 mM Tris-HCl, pH 8.2, containing 0.5 M NaCl), buffer E (20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl).

Step 1: Extraction. All operations were carried out at 0–4°C. Lyophilized mycelium (50 g) was homogenized with pestle and mortar in the presence of dry ice and sea sand and subsequently suspended in 500 ml buffer A. After stirring for 2 h, the homogenate was precentrifuged for 5 min at 5300×*g* and then centrifuged at 50 000×*g* for 30 min.

Step 2: Desalting. The supernatant containing the enzyme activity was desalted on a Sephadex G 25 column (100×6 cm, flow rate 1 ml/min), in buffer B.

Step 3: Anion exchange. The protein solution from step 2 was applied to a Q-Sepharose Fast Flow column (22×3.4 cm, flow rate 1 ml/min), which had been equilibrated with buffer B and further washed with 500 ml of the same buffer. Elution of bound protein was performed with 500 ml of 0.6 M NaCl in buffer B. Active protein fractions were concentrated by ultrafiltration using PM10 membranes (Amicon).

Step 4: Hydrophobic interaction chromatography. The protein solution resulting from step 3 was adjusted with 2 M $(NH_4)_2SO_4$ in buffer B to give a final concentration of 1 M $(NH_4)_2SO_4$. This protein solution was purified in aliquots on a phenyl-Sepharose column (11×1 cm, flow rate 0.5 ml/min), equilibrated with buffer C. After washing the gel with 30 ml of buffer C, the enzyme activity was eluted using a decreasing linear gradient of buffer C to buffer B within a total volume of 50 ml. Step 4 was repeated once with the active fractions but with a gradient of 75 ml instead of 50 ml to elute the protein.

Step 5: Gel filtration. The active protein peaks (AH-1, AH-2) derived from step 4 were separately concentrated and adjusted to buffer D. Individual samples of protein solutions were applied in separate runs on a HiLoad column HR 16/60 prepacked with Superdex 75 prep grade (Pharmacia, Uppsala, Sweden), equilibrated with buffer D and eluted with the same buffer at a flow rate of 0.5 ml/min.

Step 6: Con A chromatography. The active protein fractions of individual runs resulting f romstep 5 were combined, concentrated, adjusted to buffer E, and loaded on a Con A-Sepharose column (0.5×7 cm, flow rate 0.4 ml/min). The effluent was free of hydrolase activity. Enzyme activity was eluted with 0.5 M methyl-α-D-glucopyranoside or the corresponding mannopyranoside.

2.5. Determination of molecular mass

For estimation of the molecular mass, gel filtration was carried out on a calibrated HiLoad column HR 16/60 prepacked with Superdex 200 prep grade (Pharmacia, Uppsala, Sweden) with buffer D at a flow rate of 0.5 ml/min. The column was calibrated with the following molecular mass standards: chymotrypsinogen (25 kDa), ovalbumin (45 kDa), lipoxidase (96 kDa) and aldolase (158 kDa).

2.6. Analytical methods

Preparative native gel electrophoresis was performed with a PROTAN electrophoresis unit (BioRad, Munich, Germany) overnight at 4°C according to a modified method of Laemmli [19] on 10% running gel and 6% stacking gel.

Protein concentrations were determined according to Bradford [20] using bovine serum albumin as standard.

3. Results

3.1. Enrichment and molecular mass

A novel amidohydrolase cleaving conjugate of jasmonic acid with α-amino acids has been detected for the first time in protein preparations from the fungus *Botryodiplodia theobromae*. The enzyme was partially purified and characterized.

Table 1
Purification procedure for amidohydrolases (AH-1 and AH-2) from *Botryodiplodia theobromae*

Purification step	Protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification (fold)	Recovery (%)
1. Crude extract	892	–	–	–	–
2. Sephadex G 25	868	3715	4.28	1	100
3. Q-Sepharose	194.4	2449	12.6	3	66
AH-1					
4. Phenyl-Sepharose	30.5	1656	54.3	13	44.6
5. Superdex 75 16/60	6.7	1196	178.5	42	32.2
6. Con A-Sepharose	1.4	647	462.2	108	17.4
AH-2					
4. Phenyl-Sepharose	12.6	262	20.8	5	7
5. Superdex 75 16/60	2.8	170	60.8	14	4.6
6. Con A-Sepharose	0.55	92	167	39	2.5

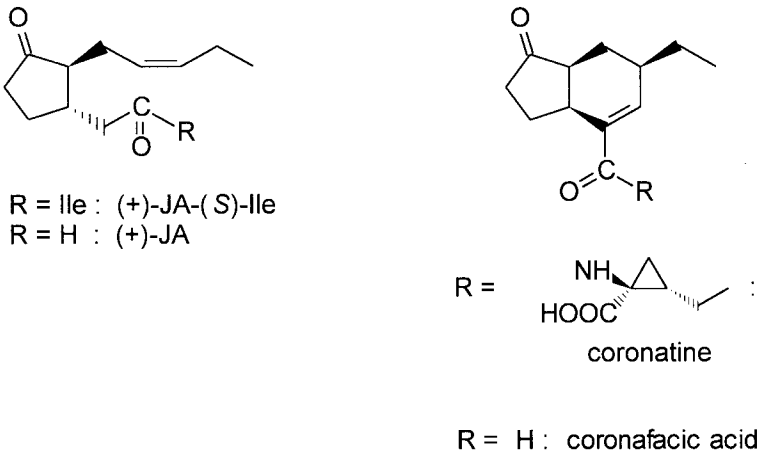

Fig. 2. Substrates other than derived from (−)-JA and their enzymic products.

Cell free extracts of the fungus were prepared from lyophilized mycelium by extraction with buffer A at 0°C and centrifugation. After desalting on Sephadex G 25 and ion exchange chromatography on Q-Sepharose Fast Flow the enzyme containing fractions were combined and applied to phenyl-Sepharose. The hydrolyzing activity was eluted over a wide range of $(NH_4)_2SO_4$ concentration (0.65–0.21 M). Rechromatograpy on phenyl-Sepharose with a flatter gradient revealed two peaks of amidohydrolase activity eluting at 0.53 M (AH-1) and 0.25 M $(NH_4)_2SO_4$ (AH-2) (Fig. 3). The steps listed in Table 1 have been proved to be suited for the purification giving a 108-fold enrichment for AH-1 and a 39-fold enrichment for AH-2. The native molecular masses of both amidohydrolases were estimated by gel filtration on Superdex 200. For both enzyme activities a molecular mass of about 107 kDa was calculated. Fig. 4 gives an example for the amidohydrolase AH-1.

Table 2
Substrate specifity of amidohydrolase AH-1 shown as specific enzyme activity given in pkat/mg protein

(−)-JA-(*S*)-Phe	112.1
(−)-JA-(*S*)-Ile	86.8
(−)-JA-(*S*)-Val	58.1
(−)-JA-(*S*)-Trp	57.2
(−)-JA-(*S*)-Leu	49.4
(−)-JA-(*S*)-AlaOMe	25.4
(−)-JA-Gly	10.0
(−)-JA-(*S*)-IleOMe	6.3
(−)-JA-(*S*)-IleOBu	6.2
(−)-JA-(*S*)-Val-(*S*)-Leu	5.2
(−)-JA-(*S*)-TyrOEt	5.8
(+)-JA-(*S*)-Ile[a]	4.5
(−)-JA-(*R*)-Phe	0
(−)-JA-(*R*)-Ile	0
(±)-JA-(*S*)-Pro	0
(±)-JA-Tyramine	0
(±)-JA-ε-Amino caproic acid	0
(±)-JA-β-Ala	0
(±)-JA-*p*-Nitranilide	0
Coronatine[a]	0

The assay mixture contained 20 nmol substrate and 28 μg AH-1 in a total volume of 100 μl of 20 mM Tris-HCl, pH 8.2. Incubation was carried out at 25°C for 30 min. (−)-JA was quantified with an enzyme-linked immunosorbent assay.
[a](+)-JA and coronafacic acid (cleavage product of coronatine) were estimated semi-quantitatively with TLC.

3.2. Stability

The partially purified amidohydrolase preparations obtained after chromatography on phenyl-Sepharose were employed to characterize the enzymes. The enzymes were stable at −20°C for at least 8 weeks and at 0–4°C for at least 5 days. Even keeping these amidohydrolase preparations at 60°C for 45 min leads to very little loss of enzyme activity. This result was consistent with temperature optima of 50°C for AH-1 and 60°C for AH-2.

The enzyme activity was completely inactivated by 1% SDS or treatment with 1 M guanidine thiocyanate for 1 h at room temperature. In contrast, both enzymes retained their activity in the presence of 6 M urea over a period of about 4 weeks at 4°C.

3.3. Protein characterization

Both amidohydrolases proved to be glycoproteins, as shown by binding of the respective enzyme activity on Con A-Sepharose. Elution of the enzyme was possible with either 0.5 M methyl-α-D-glucopyranoside or 0.5 M methyl-α-D-mannopyranoside.

3.4. Electrophoresis

Amidohydrolases AH-1 and AH-2 obtained after purification steps 1–6 were subjected to electrophoresis on a preparative native 10% polyacrylamide gel. By cutting the gel in strips of about 1 cm width and elution of the gel pieces with buffer A, the enzyme activities could be localized (Fig. 5).

3.5. pH and ion requirements

The influence of the pH on the enzyme activity was examined for amidohydrolase AH-1. This enzyme is active in the range of pH 6.2–10.9, tested in Tris-maleate-NaOH (pH 6.1–8.6), Tris-HCl (pH 7.8–9.1) and CAPS (pH 9.6–10.9). There was no distinct pH maximum.

The properties of the amidohydrolases were checked in terms of sensitivity to various metal ions like Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and Hg^{2+} at a final concentration of 1 mM and to 1 mM EDTA. The addition of metal ions or EDTA immediately to the enzyme did not have any significant effect. But a preincubation for 5 h at 4°C of the enzymes AH-1 and AH-2 with EDTA or metal ions indicated that 1 mM EDTA inhibited both activities to about 70%. On the other hand, Zn^{2+} was able to activate AH-2 leading to a 50% higher

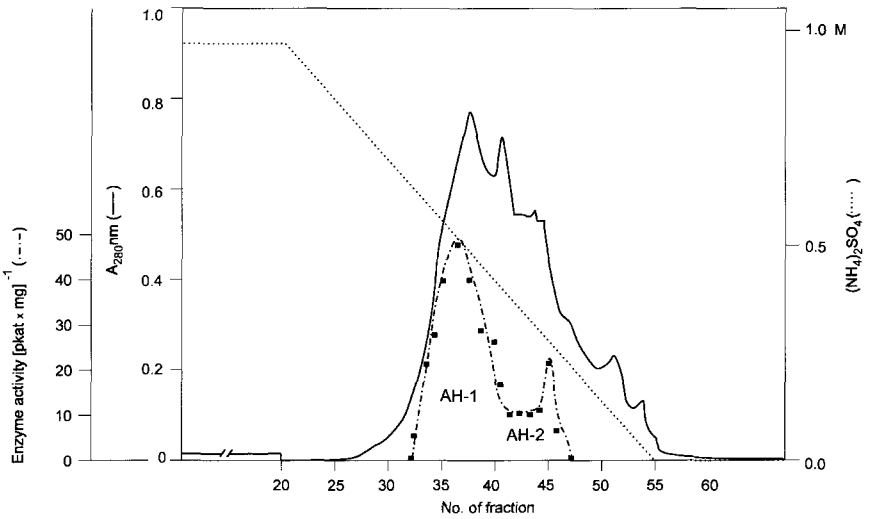

Fig. 3. Re-chromatography of pooled fractions of amidohydrolase activity on phenyl-Sepharose. The soluble protein extract was already partially purified using desalting on Sephadex G-25, anion exchange chromatography on Q-Sepharose Fast Flow and hydrophobic interaction chromatography on phenyl-Sepharose. For the re-chromatography a flat decreasing linear gradient of 1.0 M $(NH_4)_2SO_4$ in 20 M Tris-HCl, pH 8.2 to 20 mM Tris-HCl, pH 8.2 without any $(NH_4)_2SO_4$ in a total volume of 75 ml was applied. $(NH_4)_2SO_4$ concentrations of 0.53 M and 0.25 M eluted two peaks of amidohydrolase activity (AH-1 and AH-2).

activity than the control. Cu^{2+} revealed a strong inhibition of AH-1 and AH-2 of 60%, Hg^{2+} inhibited only AH-2 to 50%.

3.6. Substrate specificity

To test the specificity of the amidohydrolase, various JA-amino acid and amine conjugates were tested as substrate. These experiments were only carried out using AH-1 because of insufficient availability of AH-2 (Table 2; Figs. 1 and 2). The potential to be a substrate decreases in the order (−)-JA-(*S*)-Phe, (−)-JA-(*S*)-Ile, (−)-JA-(*S*)-Val, (−)JA-(*S*)-Trp, (−)-JA-(*S*)-Leu and (−)-JA-(*S*)-Val-(*S*)-Leu. The diastereomer (+)-JA-(*S*)-Ile (Fig. 2) turned out to be a poor substrate. JA conjugates with esters of amino acids like ((−)-JA-(*S*)-AlaOMe, (−)-JA-(*S*)-IleOMe, (−)-JA-(*S*)-IleOBu, (−)-JA-(*S*)-TyrOMe) were found to be cleaved to a very small extent, indicating the necessity of a free carboxyl group. JA conjugates with amino acids of the (*R*)-configuration ((−)-JA-(*R*)-Ile, (−)-JA-(*R*)-Phe) and JA conjugates with proline and non-protein amino acids as well as with Tyr-NH_2 and *p*-nitraniline were not cleaved at all. The phytotoxin coronatine [21], an amide of coronafacic acid (Fig. 2), which is structurally related to jasmonic acid, and 2-ethyl-1-amino cyclopropane-1-carboxylic acid (coronamic acid), was not cleaved by the enzyme.

From these experiments, the amidohydrolase seems to be

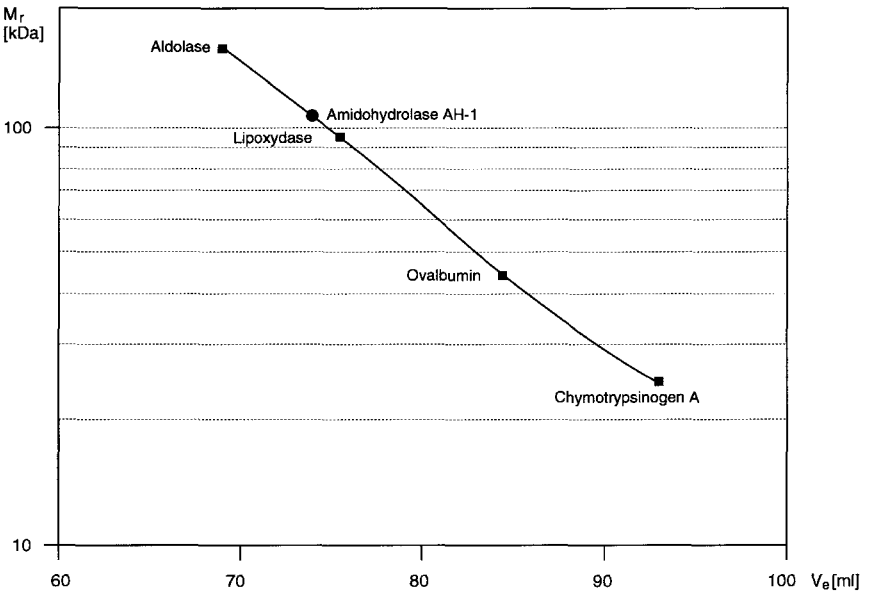

Fig. 4. Estimation of the molecular mass of the amidohydrolase AH-1 by gel filtration on Superdex 200. Partially purified enzyme was applied onto the column. From the elution volume a molecular mass of about 107 kDa was calculated.

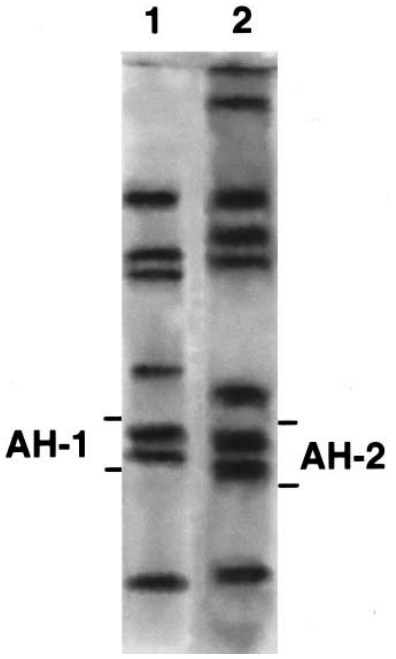

Fig. 5. Native PAGE on a 10% polyacrylamide gel of pooled fractions AH-1 (lane 1) and AH-2 (lane 2). The enzyme extracts were obtained after partial purification by anion exchange chromatography, hydrophobic interaction chromatography, gel filtration and affinity chromatography. After electrophoresis the gel was cut into pieces of 1 cm width. Enzyme activities were localized by extraction of the gel segments followed by the enzyme test.

very specific with regard to the (−)-JA moiety and certain α-amino acids with (*S*)-configuration (Phe, Ile).

4. Discussion

The fungus *Botryodiplodia theobromae* produces large amounts of secondary metabolites, among them (+)-7-iso-jasmonic acid and several minor jasmonates [13,14]. The observation that conjugates of jasmonic acid with amino acids could not be isolated from this fungus was supported by the putative presence of a mycelium-bound conjugate cleaving enzyme (Fig. 1).

Now we have detected a novel amidohydrolase, cleaving conjugates of JA with α-amino acids, in protein preparations of the fungus *Botryodiplodia theobromae* for the first time.

We achieved a 108-fold enrichment for AH-1 and a 39-fold enrichment for AH-2, by 6 purification steps.

In the purification strategy ammonium sulfate or acetone precipitation were not successful to clean up the fungal extract, because enzyme activity was observed in all fractions without any enrichment (data not shown). Chromatography on phenyl-Sepharose was proved to be an essential step for the enzyme purification resulting in two partially purified peaks of amidohydrolase activity (AH-1 and AH-2). Native PAGE of protein solutions of AH-1 and AH-2 still reveals impurities after the given purification procedure (Fig. 5). But the purification by native PAGE did not result in higher specific activity because of high losses in total enzyme activity. The binding on Con A-Sepharose characterized both amidohydrolases to be glycoproteins as shown for several amidopeptidases of animal tissue [22,23].

Gel filtration showed both enzymes to have a molecular mass of 107 kDa. For the purpose of purification the application of Superdex 75 was sufficient, but for the determination of the molecular mass it was necessary to use a Superdex 200 column.

A remarkable stability of the enzymes AH-1 and AH-2 to high temperature and in the face of denaturing agents is in accordance with results for an aminopeptidase occurring in pig kidney [24] and for a lysosomal prolyl carboxypeptidase from beef spleen [25].

Results with regard to the influence of EDTA and metal ions suggest that the amidohydrolases could be metallo-enzymes like a vacuolar aminopeptidase described by Cueva [26]. But the sensitivity of the amidohydrolases to Cu^2 and Hg^2 makes a clear classification difficult. The results suggest a complex structure of the enzyme. McDonald and Barrett [22,23] in their overview give some examples of amidohydrolases from animal tissues, which are affected by inhibitors with different effects on the enzyme molecule.

Our present results describe the amidohydrolases AH-1 and AH-2 to be very similar to each other. It would be interesting to get some data on the substrate specificity of AH-2 and to compare them with the data on AH-1. Further experiments including isoelectric focussing and a more detailed kinetic study are necessary to finally characterize the amidohydrolases from *Botryodiplodia theobromae* and to decide if AH-1 and AH-2 are real iso-enzymes.

The presence of a specific jasmonate amide hydrolyzing activity in this fungus raises the question of a possible role of this enzyme. As *Botryodiplodpia theobromae* does not produce in vitro amino acid conjugates, we would assume that the fungus may need this enzyme during the attack on the host plant for starting or modifying special plant processes, e.g. senescence, release of nutrients, beneficial for the fungal growth. The principle of jasmonate amidohydrolyzing is also realized in other fungi. Protein preparations, made from a *Cladosporium* species which was isolated from senescent anthers of *Petunia*, were also able to cleave jasmonate amides (Knöfel and Hertel, preliminary results).

There is as yet no information on a similar enzyme in plants. Our preliminary attempts to show a JA amino acid conjugate cleaving enzyme activity in plants using soluble protein fractions made from primary leaves of barley and maize failed (data not shown). Membrane-bound protein fractions of these plants were not investigated. This result strengthens the observation that JA-amino acid conjugates, especially (−)-JA-Ile, have biological activity with regard to the induction of specific proteins at least in barley [4]. Whether or not this fact is realized in other plants as well remains an open question.

Conjugates of plant hormones are discussed to be storage forms in processes regulating the active hormone level [4]. In the case of auxins, conjugation of indole-3-acetic acid (IAA) with amino acids has been shown to give a storage pool for IAA [27,28]. An IAA-Ala hydrolase from carrot has been partially purified [29] and an *Arabidopsis* ILR1 gene encodes an enzyme cleaving IAA-amino acid conjugates [30]. An IAA-L-aspartic hydrolase was induced in a bacterial strain and partially characterized [31]. The hydrolyzing enzymes are discussed to be very important in modifying the active auxin level in processes of microbe-plant interactions [32]. The enzyme activities described in this paper could point to similar mechanisms with respect to jasmonic acid.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, Grant SFB 363 and by the Ministerium für Wissenschaft und Forschung des Landes Sachsen-Anhalt, Magdeburg. The authors thank Dr. Thomas Vogt for critically reading the manuscript.

References

[1] A. Meyer, O. Miersch, C. Büttner, W. Dathe, G. Sembdner, J Plant Growth Regul 3 (1984) 1–8.
[2] O. Miersch, T. Günther, W. Fritsche, G. Sembdner, Nat Proc Lett 2 (1993) 293–299.
[3] O. Miersch, A. Preiss, G. Sembdner, K. Schreiber, Phytochemistry 26 (1987) 1037–1039.
[4] G. Sembdner, B. Parthier, Annu Rev Plant Physiol Plant Mol Biol 44 (1993) 569–589.
[5] G. Sembdner, R. Atzorn, G. Schneider, Plant Mol Biol 26 (1994) 1459–1481.
[6] C. Brückner, R. Kramell, G. Schneider, J. Schmidt, A. Preiss, G. Sembdner, K. Schreiber, Phytochemistry 27 (1988) 275–276.
[7] J. Schmidt, R. Kramell, C. Brückner, G. Sembdner, K. Schreiber, J. Stach, E. Jensen, Biomed Environ Mass Spectrom 19 (1990) 327–338.
[8] H.-D. Knöfel, G. Sembdner, Phytochemistry 38 (1995) 569–571.
[9] B.E. Cross, G.R.B. Webster, J Chem Soc C (1970) 1839–1842.
[10] O. Miersch, B. Brückner, J. Schmidt, G. Sembdner, Phytochemistry 31 (1992) 3835–3837.
[11] R. Kramell, R. Atzorn, G. Schneider, O. Miersch, C. Brückner, J. Schmidt, G. Sembdner, B. Parthier, J Plant Growth Regul 14 (1995) 29–36.
[12] Herrmann G, Kramell H-M, Kramell R, Weidhase RA, Sembdner G. In: Schreiber K, Schütte H-R, Sembdner G, editors. Conjugated Plant Hormones: Structure, Metabolism and Function. Berlin: VEB Deutscher Verlag der Wissenschaften, 1987:315–322.
[13] O. Miersch, J. Schmidt, G. Sembdner, K. Schreiber, Phytochemistry 28 (1989) 1303–1305.
[14] O. Miersch, G. Schneider, G. Sembdner, Phytochemistry 30 (1991) 4049–4051.
[15] R. Kramell, J. Schmidt, G. Schneider, G. Sembdner, K. Schreiber, Tetrahedron 44 (1988) 5791–5807.
[16] G. Schneider, R. Kramell, C. Brückner, J Chromatogr 483 (1989) 459–462.
[17] T. Albrecht, A. Kehlen, K. Stahl, H.-D. Knöfel, G. Sembdner, E.W. Weiler, Planta 191 (1993) 86–94.
[18] E. Stahl, A. Glatz, J Chromatogr 243 (1982) 139–143.
[19] U.K. Laemmli, Nature 227 (1970) 680–684.
[20] M.M. Bradford, Anal Biochem 72 (1976) 248–254.
[21] A. Ichihara, K. Shiraishi, H. Sato, S. Sakamura, K. Nishiyama, R. Sakai, A. Furusaki, T. Matsumoto, J Am Chem Soc 99 (1977) 636–637.
[22] McDonald JK, Barrett AJ. Endopeptidases. London: Academic Press, 1980:416 pp.
[23] McDonald JK, Barrett AJ. Exopeptidases. London: Academic Press, 1986:357 pp.
[24] E.D. Wachsmuth, I. Fritze, G. Pfleiderer, Biochemistry 5 (1966) 169–174.
[25] J.K. McDonald, B.B. Zeitman, S. Ellis, Biochem Biophys Res Commun 46 (1972) 62–70.
[26] R. Cueva, N. Garcia-Alvarez, P. Suárez-Rendueles, FEBS Lett 259 (1989) 125–129.
[27] Bandurski RS, Schulze A, Domagalski W. In: Schreiber K, Schütte HR, Sembdner G, editors. Conjugated Plant Hormones: Structure, Metabolism and Function. Berlin: Deutscher Verlag der Wissenschaften, 1987:11–20.
[28] Cohen JD, Slovin JP, Bialek K. In: Schreiber K, Schütte HR, Sembdner G, editors. Conjugated Plant Hormones: Structure, Metabolism and Function. Berlin: Deutscher Verlag der Wissenschaften, 1987:54–60.
[29] Cohen JD, Slovin JP, Bialek KH, Chen KH, Derbyshire MK. In: Steffens GL, Rumsey TS, editors. Biomechanisms Regulating Growth and Development. Dordrecht: Kluwer, 1988:229–241.
[30] B. Bartel, G.R. Fink, Science 268 (1995) 1745–1748.
[31] Chou JC, Kuleck GA, Cohen JD, Mulbry WW. In: 15th International Conference on Plant Growth Substances, Minneapolis. 1995:Abstract No. 156.
[32] A. Spena, J.J. Estruch, J. Schell, Curr Opin Biotechnol 3 (1992) 1959–1963.