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N-FERULOYLGLYCINE AMIDOHYDROLASE FROM BARLEY SEEDS AND ISOLATED BARLEY EMBRYOS

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Hordeum vulgare*; Gramineae; barley embryos; germination; *N*-feruloylglycine amidohydrolase; radiobiochemical TLC assay; HPLC-assay; *N*-acylamino acid.

Abstract—*N*-Feruloylglycine amidohydrolase, an enzyme catalysing the hydrolysis of a variety of *N*-acylated-L-amino acids from the cinnamoyl and substituted cinnamoyl series was isolated from barley seeds. The enzyme, which was localized within the embryo and the aleurone layer, increased in activity during the first 14 hr of seed imbibition. A 0.1 M Tris–HCl buffer pH 8.0–10% glycerol extract of germinated embryos furnished an active hydrolase preparation which was purified to *ca* 102-fold. Fractionation consisted of treatment with ammonium sulphate, gel filtration over Sepharose 6B and hydroxyapatite chromatography. Enzyme activity was measured by: (i) a radiobiochemical TLC liquid scintillation sampling method using *N*-feruloyl-2-[^{14}C]glycine-2-[^{3}H] as a substrate and (ii) an HPLC sampling assay using unlabelled *N*-feruloylglycine. The pH and temperature optima of the purified hydrolase were respectively pH 8.0 and 32°. The enzyme was very stable between pH 7.5 and 10.0, and upto a temperature of 35°. 10% Glycerol stabilized the enzyme and the activity of the crude preparation was further enhanced by 0.4 M ammonium sulphate which also provided stability during fractionation. The specific activity of the enzyme, which changed with the barley cultivar used as well as with the time of seed germination, fluctuated for the unpurified enzyme of germinated embryos from 3.6 pkat/mg (cv Zephyr; after 24 hr imbibition) to 11 pkat/mg (cv Gitane after 14 hr imbibition). The specific activity of the 102-fold purified enzyme of germinated embryos from cv Zephyr was 0.37 nkat/mg.

INTRODUCTION

The proteolytic enzymes of germinating seeds have been separated into two distinct groups, one which is already present in resting seeds and another appearing during germination [1]; there are three groups of peptidases in seeds, acid carboxypeptidases, naphthylamidases and alkaline peptidases. In addition, an *N*-acylamino acid acylase (*N*-acylamino acid amidohydrolase or acylase I (EC 3.5.1.14)) activity has also been detected in seeds and plants [2–10], bacteria* [11–18], yeast [12], fungi [19–26] and animal tissue [27–37].

During a series of investigations on the above enzymes and the related acylases: viz *N*-acyl-L-aspartic acid amidohydrolase (EC 3.5.1.15; enzyme of animal origin) [27, 31, 38, 39], acylase III† (EC 3.5.1.X; enzyme of animal origin) [32, 33, 43, 44], N^2-acetyl-L-ornithine amidohydrolase (EC 3.5.1.16; enzyme of bacterial origin) [45, 46], *N*-acyl-L-proline amidohydrolase (EC 3.5.1.-; enzyme of bacterial origin [47], N^6-acyl-L-lysine amidohydrolase (EC 3.5.1.17; enzyme of animal, fungal or bacterial origin) [29, 48] and *N*-acetyl-β-alanine amidohydrolase (EC 3.5.1.21; enzyme of animal origin) [49], mainly *N*-acylamino acids, with either acetyl, chloroacetyl or chloropropionyl moieties, have been used as substrates. Furthermore, an earlier described hippuricase of animal origin [50, 51] proved to be identical with *N*-acylamino acid amidohydrolase [28], although a more specific and inducible enzyme *N*-benzoylamino acid amidohydrolase (EC 3.5.1.32) has also been discovered in fungi [52–55] and bacteria [56, 57]. Since *N*-feruloylglycine has been identified as a sequence of barley seed (cv Union) globulins [58] and lucerne bulk leaf protein [59], the possibility of the existence of a barley seed enzyme capable of hydrolysing *N*-feruloylglycine was investigated and such an enzyme, which preferentially hydrolyses the foregoing substrate, has now been found. The enzyme, *N*-feruloylglycine amidohydrolase, N-FGAH (EC 3.5.1.X) [60], proved to be inactive on *N*-acetylglycine and other typical substrates of the above discussed acylases or amidohydrolases. In this paper the isolation of N-FGAH from barley seeds, embryos and aleurone layers as well as the purification and possible metabolic role of the enzyme will be discussed.

*Induced enzymes.

†An acylamino acid hydrolase different from acylase I, II and III has also been described [40] and this hepatitis acylase(s) has been thoroughly studied by Szewczuk and collaborators [40–42].

RESULTS AND DISCUSSION

An indication that the new enzyme occurs in barley was provided by experiments on the uptake and incorporation of *N*-feruloyl-2-[^{14}C]glycine-2-[^{3}H] by and in barley embryos. Indeed, during the latter experiments

it was clearly shown that after one hour imbibition (sterile conditions) about 22% of both radioactive tracers were taken up by the embryo tissues but that in the ribosomal fractions more ^{14}C (105 pmol per mg RNA; calculated on the basis of ferulic acid-2-[^{14}C]) than ^{3}H (70 pmol per mg RNA; calculated on the basis of glycine-2-[^{3}H]) occurred (Van Sumere C.F., Martens M. and Cottenie-Ruysschaert M., unpublished results). During the same experiments it was further observed that certain inhibitors such as cycloheximide (1 mM) affected the incorporation of ^{3}H (27.3% inhibition) more than the incorporation of ^{14}C (12.8% inhibition). Thus some of the double labelled material was split before each of the components of *N*-feruloyl-2-[^{14}C]glycine-2-[^{3}H] were again, at least in part, separately incorporated into the ribosomal fraction of barley embryos. The new enzyme has been obtained (see Experimental) and two methods: a radiochemical TLC assay (Fig. 1) and an HPLC assay (Fig. 2), which both give comparable results (Fig. 3) have been developed for the determination of its activity. Both the embryo and the aleurone layer contain N-FGAH activity but no such enzyme could be obtained from the endosperm (Table 1). Furthermore, the evolution of the enzyme activity of excised barley embryos, with the time

Table 1. Distribution of N-FGAH activity (unpurified S_{105} fraction) in ungerminated and germinated barley seeds, embryos, aleurone layers, endosperm, shoots and roots

Fraction investigated	Specific activity (pkat/mg protein)
A. Barley cv Zephyr	
Ungerminated seeds	6.4
Ungerminated embryos	3.7
Germinated embryos (24 hr imbibition)	3.6
Aleurone layers (72 hr imbibition)	3.8
Endosperm (72 hr imbibition)	0.0
Shoots (72 hr imbibition)	0.1
Roots (72 hr imbibition)	0.0
B. Barley cv Gitane*	
Ungerminated embryos	7.2
Germinated embryos (14 hr imbibition)	11.0
Germinated embryos (24 hr imbibition)	7.3

*Although cv Zephyr has been mainly used during this work, cv Gitane contained a higher specific activity. However, difficulties with the seed supply were responsible for the selection of cv Zephyr.

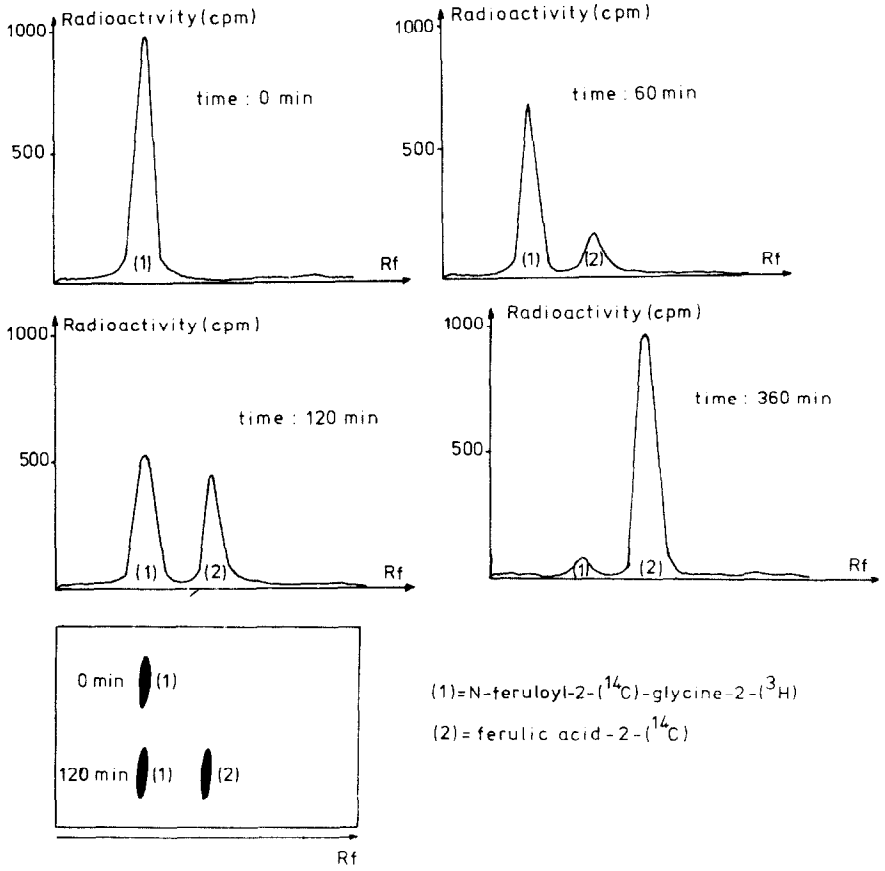

Fig. 1. The hydrolysis of *N*-feruloyl-2-[^{14}C] glycine-2-[^{3}H] with N-FGAH (unpurified S_{105}-fraction originating from germinated and excised barley embryos) (Reaction mixture: 2 ml S_{105}; 0.5 ml 0.876 mM substrate; pH 8.0, temp. 30°). With time 100 μl of the reaction mixture were spotted on a thin layer, chromatographed and scanned as described. (For autoradiography Agfa Structurix D7 film was employed.)

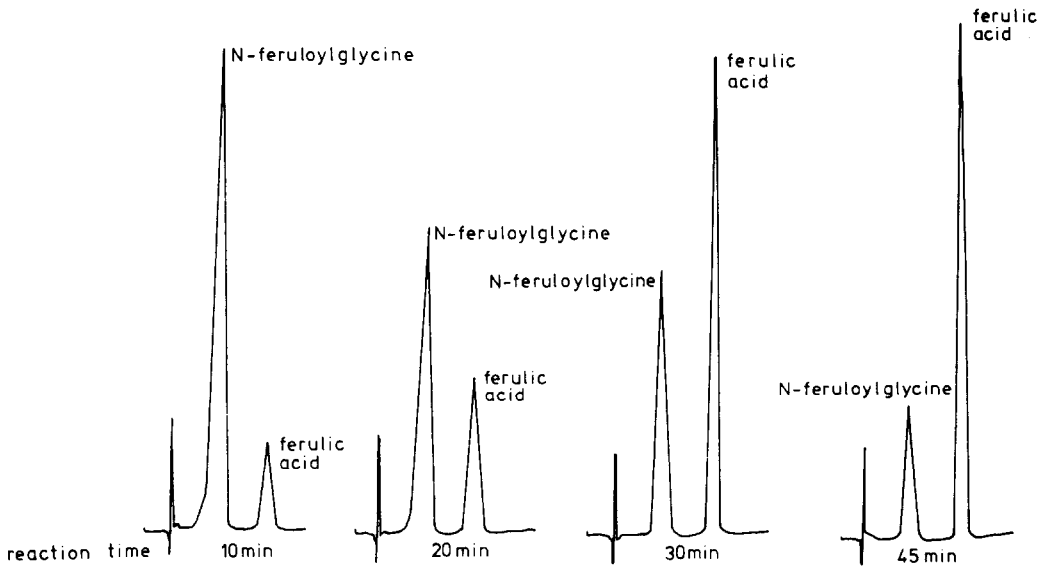

Fig. 2. HPLC-assay of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction) (For conditions used see Experimental).

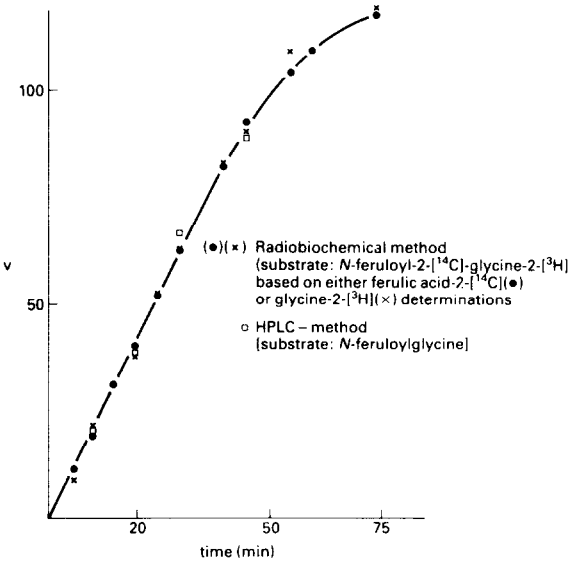

Fig. 3. Progress curve of N-FGAH from germinated and excised barley embryos (EC 3.5.1.X) (purification: 102-fold) with either double labelled (radiobiochemical TLC assay) or unlabelled (HPLC-assay) *N*-feruloylglycine as substrate (the reaction velocity (v) is expressed in nmol ferulic acid or glycine produced/0.1 mg protein).

Table 2. Relationship between specific activity of N-FGAH (EC 3.5.1.X) of excised barley embryos (cv Gitane) and time of seed imbibition

Time of imbibition (hr)	Specific activity (pkat/mg protein)
0	7.2
1	7.7
2	8.2
4	9.3
6	8.8
8	9.8
10	10.0
12	10.3
14	11.0
16	9.8
18	9.0
20	7.6
22	7.0
24	7.3

of seed germination, reached a maximum around 14 hr imbibition (Table 2). In Table 3 the presence of the N-FGAH in a few plants is represented. So far the highest activity has been found in germinated barley embryos although weak enzyme activities have been detected in a few other species. The amidohydrolase activity varies in barley with the cultivar studied. Indeed, barley cv Gitane contained almost twice the activity of cv Zephyr (see Table 1).

The purification of the amidohydrolase isolated from germinated (24 hr) and excised barley embryos is further summarized in Table 4. Ion exchange chromatography using DEAE-Sephadex A_{50} or affinity chromatography using an AH-Sepharose 4B matrix and *N-p*-hydroxybenzoylglycine* as a ligand proved unsuccessful. In addition employment of corning pore glass to which the latter

N-p-Hydroxybenzoylglycine is only poorly hydrolysed by the enzyme.

Table 3. Comparison of the specific activity of N-FGAH (unpurified S105-fraction) obtained from different plants

Plant species	Specific activity (pkat/mg protein)
Germinated (24 hr) barley embryos (*Hordeum vulgare*)	3.6
Germinated (24 hr) rye embryos (*Secale cereale*)	0.8
Germinated (24 hr) pea embryos (*Pisum sativum*)	0.2
Spinach leaves (*Spinacia oleracea*)	0.2
Lettuce leaves (*Lactuca sativa*)	0.2
Clover leaves and stems (*Trifolium sp.*)	0.1
Lucerne leaves and stems (*Medicago sativa*)	0.0
Germinated (24 hr) sunflower embrvos (*Helianthus annuus*)	0.0

ligand is attached through a stabilized diazonium salt as well as hydrophobic interaction chromatography failed. Purification of the enzyme present in the acetone extract from entire and ungerminated barley seeds was much less successful. Indeed, an ammonium sulphate treatment combined with gel chromatography over Sepharose 6B resulted only in a 20-fold purification. For this and other reasons this study was mainly performed with the enzyme originating from excised barley embryos.

The pH and temperature optima of the purified enzyme were respectively pH 8 and 32° [the optimal conditions of the crude enzyme (S_{105} fraction) being pH 8 and 35°]. The relationship between the amount purified enzyme and the initial velocity proved further to be linear for a final enzyme concentration ranging between 0.05 and 0.21 mg protein per ml. N-FGAH proved to be unstable below pH 5 and above 35°. The enzyme could further be stabilized by the addition of 10% glycerol and an enzyme preparation so treated could be kept at −28° for several months without serious loss of activity. Furthermore, addition of 0.4 M ammonium sulphate enhanced the activity of the unpurified S_{105} solution by 55% but no such effect was noticeable with the purified enzyme, although 0.1 M ammonium sulphate additions were required during chromatographic purification. This beneficial effect can be ascribed to the sulphate ion, since 0.4 M potassium sulphate also increased the activity of the unpurified enzyme by 36%. N-FGAH increases in activity rather sharply during the first 14 hr (maximum activity) of germination and this fact, in conjunction with the finding that almost all the activity is concentrated in the embryo and the aleurone layer, indicates a role for the enzyme during germination. In association with proteinases and exo- and endopeptidases, the amidohydrolase may be responsible for the hydrolysis of the *N*-feruloylglycyl-moieties of at least certain reserve proteins, because such *N*-feruloylglycine-containing proteins occur in barley seeds [58]. An analogous degradation has also been proposed for the *N*-acylated proteins of animal origin. Moreover, the suggested degradation of the *N*-feruloylglycine-containing proteins would fit well with Mikola's ideas [1] for the enzymatic degradation of the reserve proteins of barley endosperm. During the first stage the insoluble reserve proteins would be converted by proteinases and acid carboxypeptidases into a mixture of small peptides and amino acids. Subsequently, the amino acids and peptides would be absorbed by the scutellum, which is attached to the embryo [61 65]. Finally, the residual peptides and also those containing ferulic acid could be hydrolysed in the scutellum by neutral and (or) alkaline peptidases and amidohydrolases, amongst them N-FGAH. An analogous system for the hydrolysis of the reserve proteins of the protein bodies [66, 67] of the aleurone layer has also been suggested by Mikola [1].

EXPERIMENTAL

Plant material. Barley [*Hordeum vulgare*; cv Zephyr (or where indicated cv Gitane)] originating from the test fields of the European barley convention at Proven (West Flanders, Belgium) was used throughout this work.

Germination of the seeds and sterile excision of the embryos. Sets of 12000 barley seeds were first washed with 1% NaOCl and sterile H_2O and then spread in groups of 100 in plastic boxes (15 × 20 cm) provided with sterile Whatman no. 1 paper. After addition of 10 ml sterile H_2O the seeds were germinated in the dark, at 20° and nearly 90% humidity, in a controlled environment room (Brabender OHG, Duisburg, F.R.G.). The environment room was first made sterile by UV irradiation for 24 hr. After 24 hr germination the embryos were excised by scalpel. Although the activity of N-FGAH reached a maximum

Table 4. Purification of N-FGAH isolated from germinated (24 hours) and excised barley embryos

Fractions	Volume (ml)	Total protein (mg)	Total enzyme activity (nkat)	Specific activity (pkat/mg) protein)	Activity recovery (%)	Purification factor
I Extract (S_{105}–fraction)	850	3740.0	13.6	3.6	100	1
II $(NH_4)_2SO_4$ fraction (30–35%)	120	675.0	12.4	18.4	91	5
III Gelchromatography (Sepharose 6B)	21	75.6	10.9	144.6	81	40
IV Hydroxyapatite chromatography	5.5	17.1	6.3	367.3	46	102

at *ca* 14 hr imbibition and even though ungerminated embryos proved to possess fair enzyme activity, the 24 hr period was chosen for practical convenience.

Control of the sterility of the reaction mixtures. The extracts were plated in petri dishes on a sterile medium consisting of 2% agar, 1% yeast extract, 0.1% KH_2PO_4 and 2.5% glucose. After 3 days at 30° only a few colonies developed in the plated extracts which were prepared without any special sterility precautions.

Preparation of aleurone layers of barley. The isolated aleurone layers were prepared according to the procedure of ref. [68].

Substrates. For the synthesis of *N*-feruloyl-2-[^{14}C]glycine-2-[^{3}H] (specific activity [^{14}C]: 5.92 MBq/mmol; [^{3}H]: 22.57 MBq/mmol) both the mixed anhydride method [69] and the active ester method [70] have been used. Unlabelled *N*-feruloylglycine was synthesized as described in ref. [70].

Radioactivity counting. Radioactivity was counted by means of a Packard Tricarb Model 3380 liquid scintillation spectrometer (provided with an absolute activity analyzer Model 544) using 10 ml Instagel (Packard). TLC-scanning was performed with a Berthold (Wildbad, F.R.G.) thin-layer scanner (flow counter).

Preparation of the enzyme extract and purification of the enzyme

N-FGAH extract from barley embryos. 1000 excised embryos originating from germinated barley seeds were first ground in a cold mortar, with 10 ml 0.1 M Tris–HCl buffer pH 8.0 containing 10% glycerol, and then further homogenized with a Potter–Elvejhem apparatus. The homogenate was centrifuged for 15 min in the cold in a Spinco centrifuge at 22000 *g* and the supernatant was recentrifuged at 4° for 120 min at 105000 *g*. The remaining supernatant, which contained the enzyme, will be called barley embryo fraction S_{105} (unpurified enzyme extract).

Enzyme purification. $(NH_4)_2SO_4$ *fractionation.* Fraction S_{105} was subjected to $(NH_4)_2SO_4$ treatment at pH 8. The material precipitating between 30 and 35% saturation was dissolved in 0.1 M Tris–HCl buffer (pH 8) containing 10% glycerol and the solution obtained was further purified by chromatography on Sepharose 6B.

Chromatography on Sepharose 6B. Sepharose 6B (Pharmacia, Uppsala Sweden) was equilibrated at 5° in 0.1 M Tris–HCl buffer pH 8, containing 10% glycerol and 0.1 M $(NH_4)_2SO_4$. A 15 ml sample of the 30–35% $(NH_4)_2SO_4$ ppt. of fraction S_{105}, dissolved in the above buffer system and containing *ca* 85 mg protein, was then charged onto a Pharmacia K26/70 (60 cm × 2.6 cm) column. Elution of the protein was performed with the same buffer under constant hydrostatic pressure (49 cm H_2O) and at 5°. Under these conditions the flow rate was 2.3 ml/cm^2/hr. 5 ml fractions were automatically collected and those showing enzyme activity were pooled and again treated with $(NH_4)_2SO_4$. Finally the protein fraction precipitating between 35 and 45% $(NH_4)_2SO_4$ was isolated and dissolved in 6.5 ml 0.1 M Tris–HCl buffer (pH 8) containing 10% glycerol.

Chromatography on hydroxyapatite. 25 g hydroxyapatite (Biogel HTP BioRad-California) were suspended in 0.05 M Pi buffer pH 6.8 containing 10% glycerol and 0.05 M $(NH_4)_2SO_4$. Several samples of the Sepharose 6B purified enzyme were pooled to *ca* 40 ml and then charged on a Pharmacia K26/40 (13.5 × 2.6 cm) column. For the latter purpose a syringe connected to a three-way valve, which was combined with the top A26 adaptor system of the column, was employed. Elution was performed, at 5°, with the following gradient system: (a) 210 ml 0.05 M Pi buffer pH 6.8 containing 10% glycerol and 0.05 M $(NH_4)_2SO_4$; (b) 300 ml 0.30 M buffer pH 6.8 containing the same amounts of glycerol and $(NH_4)_2SO_4$ and (c) 150 ml 1 M NaCl. The elution speed (peristaltic pump) was 42.4 ml/hr and 5.0 ml fractions (detection at 280 nm), were automatically collected.

N-FGAH extract from barley seeds. Barley seeds were first dehusked by mixing the seeds at medium speed in a Waring blender. The husks were blown away and the seeds were ground to a fine powder.

Subsequently, 50 g of the barley flour were suspended in 125 ml cold (−15°) Me_2CO and then mixed for 10 min. After centrifugation (5 min) at 1700 g the supernatant was discarded and the precipitate once more treated in the same way. Following the second centrifugation the residue was suspended in 75 ml cold (−15°) Et_2O for 2 min and the suspension was again centrifuged. After removal of the supernatant an acetone powder was obtained by drying the precipitate in the cold (*ca* 4°) on Whatman no. 1 paper. The acetone powder was then suspended in 125 ml 0.10 M Tris–HCl buffer pH 8.0 containing 10% glycerol, 0.1 M $(NH_4)_2SO_4$ and 1 g PVP (2%). After extraction for 60 min with a Vibromix apparatus (El N°10049 Chemap AG Männedorf, Switzerland) the suspension was centrifuged at 1700 g for 5 min. The ppt. was discarded and the supernatant filtered over gauze. After a second addition of 1 g PVP and centrifugation at 22000 *g* for 15 min, the ppt. was discarded and the supernatant filtered over gauze. Finally after renewed addition of PVP and centrifugation for 60 min at 105000 *g*, the supernatant was filtered over filter paper resulting into the S_{105} barley fraction (different from the above germinated barley embryo fraction S_{105}).

Protein determination. Protein was assayed according to the method of ref. [71].

TLC on silica gel–cellulose (1:1; w/w) layers was performed as described earlier (72, 73) using toluene, ethyl formate, formic acid (T.E.F. 5:4:1). The compounds (R_f glycine: 0.00; R_f: *N*-feruloylglycine: 0.25; R_f ferulic acid: 0.52) were detected by examination of the dried chromatograms under UV light (360 nm) before and after spraying the thin layers with 2 M NaOH. Further information on the R_f values and the appearance of the compounds studied in UV light and (or) after spraying with diazotized *p*-nitroaniline or treatment with Gaffney's reagent are given in ref. [73–75].

Enzyme assay. Radiobiochemical TLC assay. The radioactive assay for N-FGAH consisted normally of equilibrating: 50 μl *N*-feruloyl-2-[^{14}C] glycine-2-[^{3}H] (5 mM), 100 μl enzyme (purification: 102-fold; 2.1 mg protein/ml) and 850 μl 0.1 M Tris–HCl buffer (pH 8) at 30° (Final substrate and enzyme concentration: 0.25 mM and 0.21 mg protein*). Periodically (from 0 to 90 min) 100 μl of the reaction mixture were spotted on silica gel–cellulose (1:1; w/w) layers and the spots were immediately dried by means of a stream of hot air (enzyme destruction). Subsequently the thin layers were treated with water vapour [72, 73] and chromatographed with T.E.F. (5:4:1). After scanning of the thin layer with a flow counter (Berthold scanner) the ferulic acid-2-[^{14}C] spots were removed from the plate and counted by means of a liquid scintillation counter. From the specific activity of the ferulic acid or glycine moiety of the substrate the amount ferulic acid-2-[^{14}C] or glycine-2-[^{3}H] liberated per 0.1 mg protein (v) was calculated and plotted against time (progress curve). Analogous procedures were employed when other labelled substrates were tested (see the following paper).

HPLC assay. The reaction mixture equilibrated at 30° consisted of: 25 μl *N*-feruloylglycine (5 mM), 50 μl enzyme (2.1 mg protein/ml) and 425 μl Tris–HCl buffer (0.1 M; pH 8). Periodically 100 μl of the above mixture were injected in micro sealed

*In the case of unpurified enzyme the final protein concentration was 1.05 mg per ml.

vials and boiled for 10 min (enzyme destruction). From the content of each vial 50 μl were analysed by means of the gradient HPLC-system described in ref. [73]. The HPLC-equipment (Hewlett Packard Liquid Chromatograph 1084B equipped with a Pye Unicam UV detector LC-3) was calibrated with known amounts of ferulic acid and *N*-feruloylglycine (or analogous compounds, see the following paper). The progress curve was obtained as described above (see radiobiochemical assay procedure).

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