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CHARACTERISTICS AND SPECIFICITY OF PURIFIED *N*-FERULOYLGLYCINE AMIDOHYDROLASE FROM 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; substrate specificity; *N*-acylamino acids ; enzymic characteristics; inhibitors.

Abstract—*N*-Feruloylglycine amidohydrolase with an estimated M_r of 155 000, showed with *N*-feruloylglycine at pH 8 and 30° a K_m of 85 μM, a V_m of 3.92 nmol/0.1 mg protein/min, a physiological efficiency (V_m/K_m) of $46.1.10^3$ and an apparent activation energy of 43.5 kJ/mol. Sulphydryl reagents were shown to decrease enzyme activity and relative high concentrations (10 mM) of metal chelating reagents (EDTA, dithizone and *o*-phenanthroline) were also inhibitory. The effect produced by *o*-phenanthroline could be partially reversed by $CuCl_2$ and $CoCl_2$ and to a lesser extent by $ZnCl_2$ and $MnCl_2$. *N*-Feruloyldipeptides such as *N*-feruloylglycyl-L-phenylalanine ($K_i=42$ μM; $\alpha=7.8$ and $\beta=0$) and *N*-feruloylglycyl-L-leucine ($K_i=300$ μM; $\alpha=5.8$; $\beta=0$) were potent reversible mixed type inhibitors. The purified enzyme did not show any deformylase or amidase activity and was thus totally different from *N*-acylamino acid amidohydrolase (EC 3.5.1.14). In addition, the pseudopeptide bond of *N*-feruloylglycine was not split by a series of peptidases and proteinases. *N*-Feruloylglycine amidohydrolase is a new acylase of plant origin and the name proposed for the enzyme is well supported by substrate specificity studies.

INTRODUCTION

In an accompanying paper the detection and purification of *N*-feruloylglycine amidohydrolase (N-FGAH) from germinated barley embryos has been described [1]. In this paper further information on the enzymic characteristics of N-FGAH as well as inhibitor studies with sulphydryl reagents, metal chelating agents and mixed type peptide inhibitors, will be presented. In addition, the specificity of the new plant enzyme and a comparison with other acylases and peptidases will be discussed.

RESULTS AND DISCUSSION

N-Feruloylglycine amidohydrolase (N-FGAH) was isolated and purified from barley embryos as described [1]. The K_m value of the purified (102-fold) N-FGAH with *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] as substrate was $K_m=86$ μM (Lineweaver–Burk plot [2]) or $K_m=84$ μM (Eadie–Hofstee plot [3, 4]). The latter values (average value: 85 μM) agree rather well, although the Eadie–Hofstee plot is considered to be the most accurate [5, 6]. The V_m values obtained with the above plots were respectively 3.95 and 3.88 nmol ferulic acid-2-[^{14}C] formed (or the equivalent amount of substrate split) per 0.1 mg protein per min; the corresponding physiological activities (V_m/K_m) being $45.9.10^3$ and $46.2.10^3$. Additional studies proved that the enzymatic reaction followed first order kinetics (Fig. 1) between 20 and 32°.

The apparent activation energy of the enzyme calculated via Arrhenius plots (Fig. 2) for the same temperature intervals of Fig. 1 showed that $E_A=43.5$ kJ/mol. This value is in good agreement with the apparent activation energy $E_A=42.7$ kJ/mol calculated via the temperature coefficient Q10 [7].

A survey of the effect of some common sulphydryl reagents on N-FGAH is shown (Table 1). This Table, which represents the minimal concentration of reagent at which 100% enzyme inhibition occurred, clearly indicates that N-FGAH is very sensitive to sulphydryl blocking reagents such as mercurichloride and *p*-chloromercuribenzoate. *N*-Ethylmaleimide, which forms addition products with SH-groups was also fairly active. However, disodium maleate did not show any inhibitory effect. The latter result may be due to steric factors or electrostatic influences [8]. Disulphide reducing or sulphydryl oxidizing reagents were only active at higher concentrations. Nevertheless, the above experiments seem to indicate that sulphydryl groups play an important role during catalysis.

Amongst the inhibitors which are normally considered to affect metal containing groups, which may be of importance for either enzyme action or enzyme stability, only 10 mM concentrations of EDTA (61% inhibition), diphenyl-1,5-thiocarbazone (dithizone; 79% inhibition) and *o*-phenanthroline (97% inhibition) proved to be strongly inhibitory. *o*-Phenanthroline produced already a 29% inhibitory effect at 1 mM but a 10-fold smaller concentration was inactive. In addition, the inhibition produced by 2.5 mM *o*-phenanthroline (73% inhibition) could be totally reversed by 0.66 mM $ZnCl_2$. The same effect could also be obtained by the addition of $CuCl_2$, $CoCl_2$ and to a lesser extent also by nickel and manganous ions. Bivalent cations are thus of importance for the proper function or the stability of N-FGAH. Similarly Lugay and Aronson [9] found that Co^{2+} enchanced the aminoacylase from Paloverde (*Parkinsonia aculeata*) seeds.

In addition to the above 'classical inhibitors', the effect of some reversible inhibitors of the N-FGAH of germina-

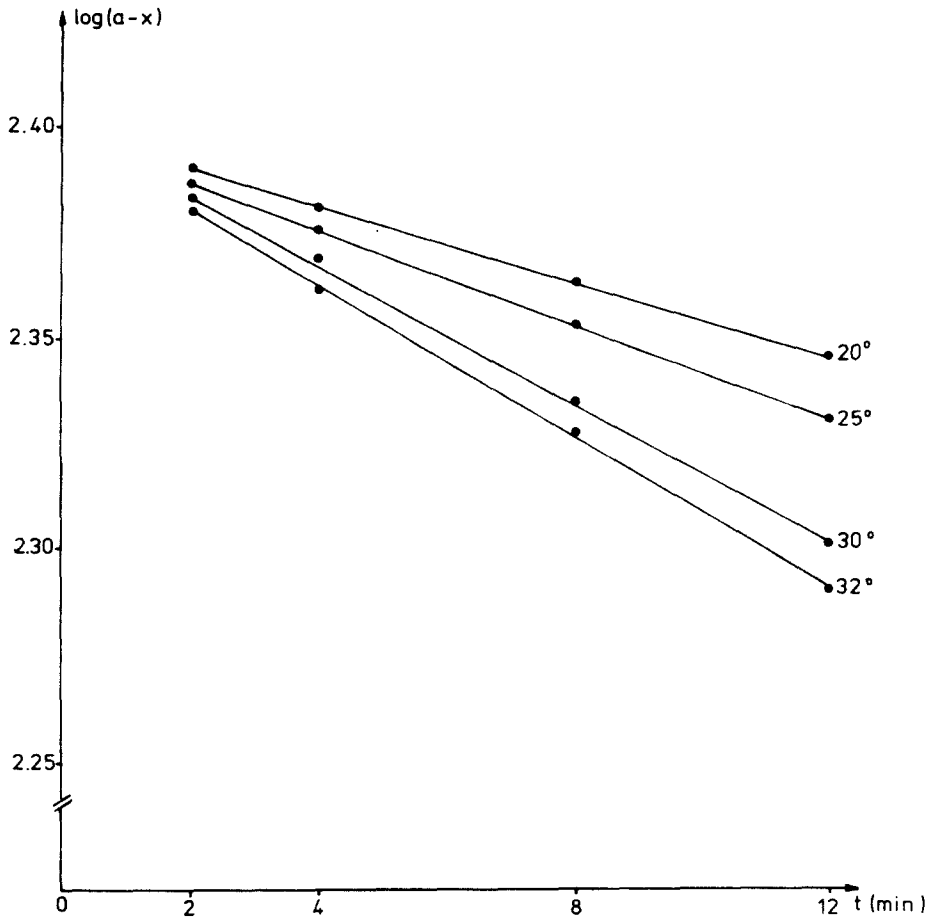

Fig. 1. Graphical proof for first order reaction of the N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction) with *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] as a substrate. a: Concentration of the substrate at $t=0$ (0.25 mM); x: amount of substrate hydrolysed (or amount of ferulic acid-2-[^{14}C] produced) at time t.

ted barley embryos, such as *N*-feruloyl-Gly-L-PheOH (5 mM) and *N*-feruloyl-Gly-L-LeuOH (5 mM), has been investigated. The reversible inhibitory character of the latter compounds was established by Sephadex G-25 separation of the enzyme from the *N*-feruloyl-Gly-L-PheOH (or *N*-feruloyl-Gly-L-LeuOH) substrate mixture (see also Dixon and Webb [7]). Indeed, subsequent testing proved that the enzyme activity could be to a large extent (*ca* 85%) recovered proving that the inhibition was reversible. Subsequently the type of inhibition (competitive, noncompetitive, mixed type, etc.) was investigated and proven via kinetic studies [10] to be of the mixed type (see Fig. 3).

In addition, the K_i value for *N*-feruloyl-Gly-L-PheOH was found to be 43 μM (Hanes plot [11]) or 40 μM (Dixon plot [12], Fig. 4), which results in an average K_i value of 42 μM. This average K_i value, which represents the reciprocal of the enzyme inhibitor affinity indicates further that the inhibitor is rather effective, especially when the K_m value (85 μM) for the substrate *N*-feruloyl-GlyOH is taken into consideration. For the same inhibitor the α-values obtained via either Lineweaver–Burk [2], Hanes [11] or Cornish-Bowden [13] plottings (Fig. 5) were on average found to be 7.8. This means that α is situated between 1 and ∞, which furnishes additional proof for the occurrence of a mixed type inhibition ($1 < \alpha < \infty$; $\beta=0$) (Webb [10]).

In the case of *N*-feruloyl-Gly-L-LeuOH the average K_i value proved to be 0.3 mM. This indicates that the latter substance is a less efficient inhibitor for N-FGAH than *N*-feruloyl-Gly-L-PheOH. In addition an α value of 5.8 was found which shows that as a mixed type inhibitor *N*-feruloyl-Gly-L-LeuOH tends even more strongly towards the totally non-competitive type of inhibitor ($\alpha=1$, $\beta=0$) [10] than *N*-feruloyl-Gly-L-PheOH.

The purified (102-fold) N-FGAH exhibited a rather wide range of substrate specificities and in a search for the most efficiently hydrolysed substrate the K_m, V_m and especially the 'physiological efficiency' (V_m/K_m) (14) of the enzyme with several different *N*-acylamino acids were compared (Table 2). The enzyme in fact shows the greatest relative affinity and 'physiological efficiency' with *N*-feruloylglycine as a substrate. The 'physiological efficiency' decreases with the degree of substitution of the aromatic ring of the cinnamoyl moiety as well as with the reduction of *N*-feruloylglycine to dihydroferuloylglycine,

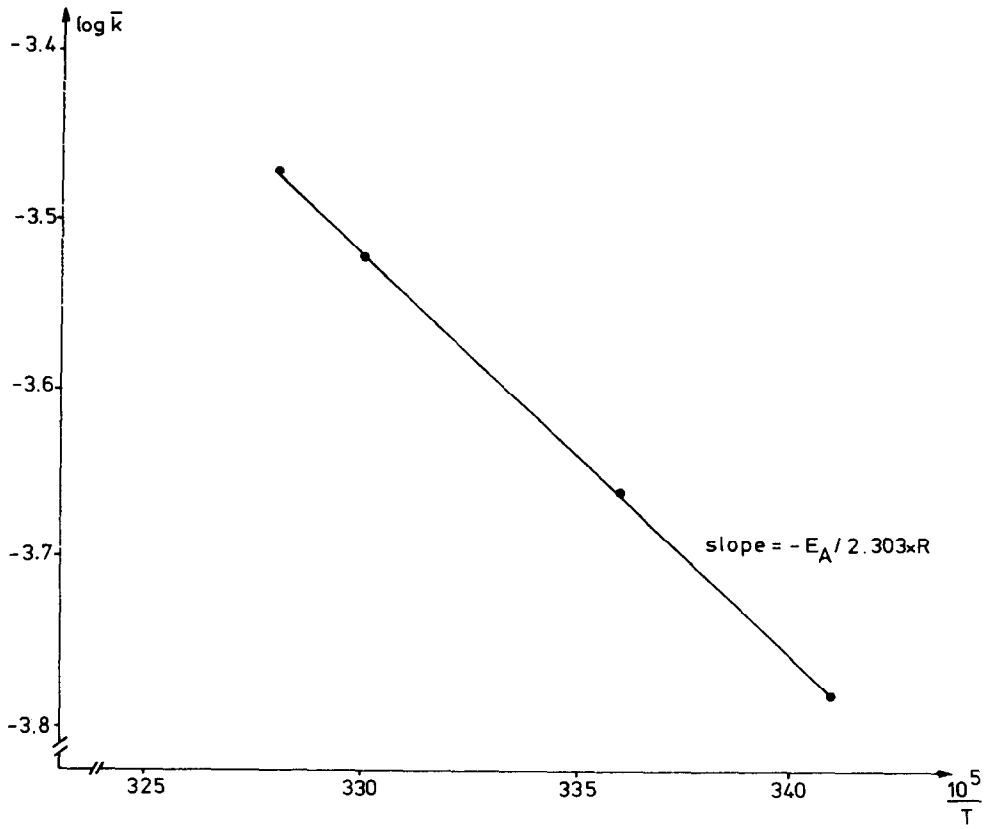

Fig. 2. Arrhenius plot for the calculation of the apparent activation energy of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction); substrate: *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H](k = average first order rate constant).

Table 1. Effect of sulphydryl reagents on the activity of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction) with *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] as a substrate (the table shows the smallest concentrations of reagents producing 100% inhibition)

Inhibitor	Minimal concentration producing 100% inhibition
SH-group oxidizing reagents	
o-Iodosobenzoate (sodium salt)	5 mM
cystine	not inhibitory
Mercaptide forming reagents	
Mercurichloride	0.005 mM
p-chloromercuribenzoate (sodium salt)	0.05 mM
SH-group alkylating reagents	
Iodoacetate (sodium salt)	4 mM
iodoacetamide	7.5 mM
Reagents forming addition products with SH-groups	
N-Ethylmaleimide	1 mM
maleate (disodium salt)	not inhibitory
Disulphide reducing reagents	
1,4-Dithiothreitol	2 mM
2-mercaptoethanol	20 mM
L-cysteine	50 mM
reduced glutathione	50 mM

although sinapoylglycine which contains a more heavily substituted aromatic ring than cinnamoylglycine is more slowly hydrolysed than the latter compound. In addition, also the amino acid moiety of the substrates seems to be of importance since *N*-feruloyl-L-alanine is much more slowly hydrolysed than *N*-feruloylglycine. All benzoylglycine derivatives are further hydrolysed with a much lower 'physiological efficiency' (*ca* 15 times smaller) than the corresponding cinnamoyl derivatives.

It is also remarkable that the new enzyme did not hydrolyse any of the substrates which are normally used for the determination of the activity of other amidohydrolases (acylases, deformylases and amidases) (see Table 3). Indeed, the purified N-FGAH does not show any deformylase nor a *N*-feruloylamide amidohydrolase (amidase) activity. With regard to substrate specificity (Table 3) of the represented acylases (inclusive of those represented in Table 4), the barley enzyme shows a totally different substrate specificity. Indeed, although *N*-FGAH splits *N*-benzoyl-1-[^{14}C] -glycine-2-[^{3}H] (double labelled hippuric acid) after five hr for *ca* 30%, the latter enzyme must be considered to be different from *N*-benzoylamino acid amidohydrolase, because in contrast with hippuricase, which splits *N*-acetyl-L-amino acids (at least to certain extent [18]), N-FGAH does not show the slightest activity with any of the *N*-acetylamino acids tested (Table 3).

The opposite holds true to a large extent for the *N*-acylamino acid amidohydrolase (EC 3.5.1.14) from hog kidney. Indeed, from Table 5 it follows that *N*-acetylglycine is a good substrate for this enzyme and that *N*-

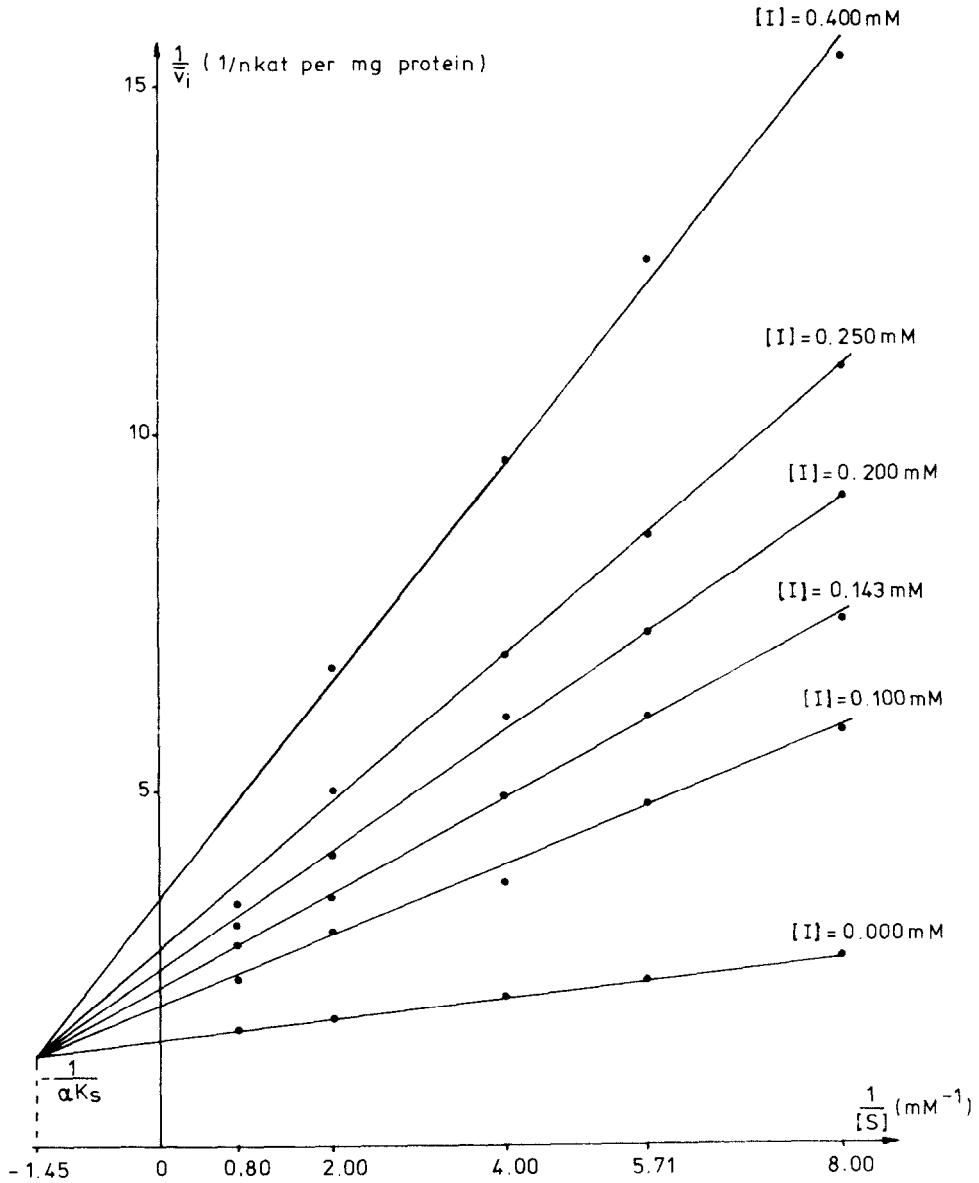

Fig. 3. Lineweaver–Burk plot of the mixed type inhibition of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction) produced by *N*-feruloyl-Gly-L-PheOH (substrate used: *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H]; radiochemical TLC method).

benzoylglycine and *N*-cinnamoylglycine are much less easily split while on a relative basis none of the substituted *N*-cinnamoyl- or *N*-benzoylglycine compounds are readily hydrolysed.

The activity of the acylase of animal origin is higher with the *N*-acylglycine substrates of the benzoyl series than with the substrates of the corresponding cinnamoyl type. The reverse holds true for the plant enzyme. Indeed, the specific activity of the N-FGAH with *N*-feruloylglycine (367.3 pkat/mg protein [1]) is *ca* 230 times higher than that of the amino-acylase from hog kidney (1.60 pkat/mg protein; see Table 5).

Since N-FGAH seemed to be different from all other analogous pseudopeptide splitting enzymes (e.g. acylases, deformylases and amidases (Tables 3–5) it was also decided to investigate the possible relationship of the former enzyme with other peptide bond hydrolysing enzymes such as exo- and endopeptidases. Mikola [19] classified the proteolytic barley enzymes in five groups, viz: two groups of endopeptidases and three groups of exopeptidases (amongst them carboxypeptidases, naphthylamidases and peptidases). The naphthylamidases and the peptidases (pH optimum 8-10) seemed to be rather interesting to investigate. For this reason the possible activity of N-FGAH with Azocoll (a substrate for endopeptidases [20]) was studied. In addition, the possible carboxypeptidase activity of the N-FGAH preparation with a series of suitable substrates (such as: *N*-carboben-

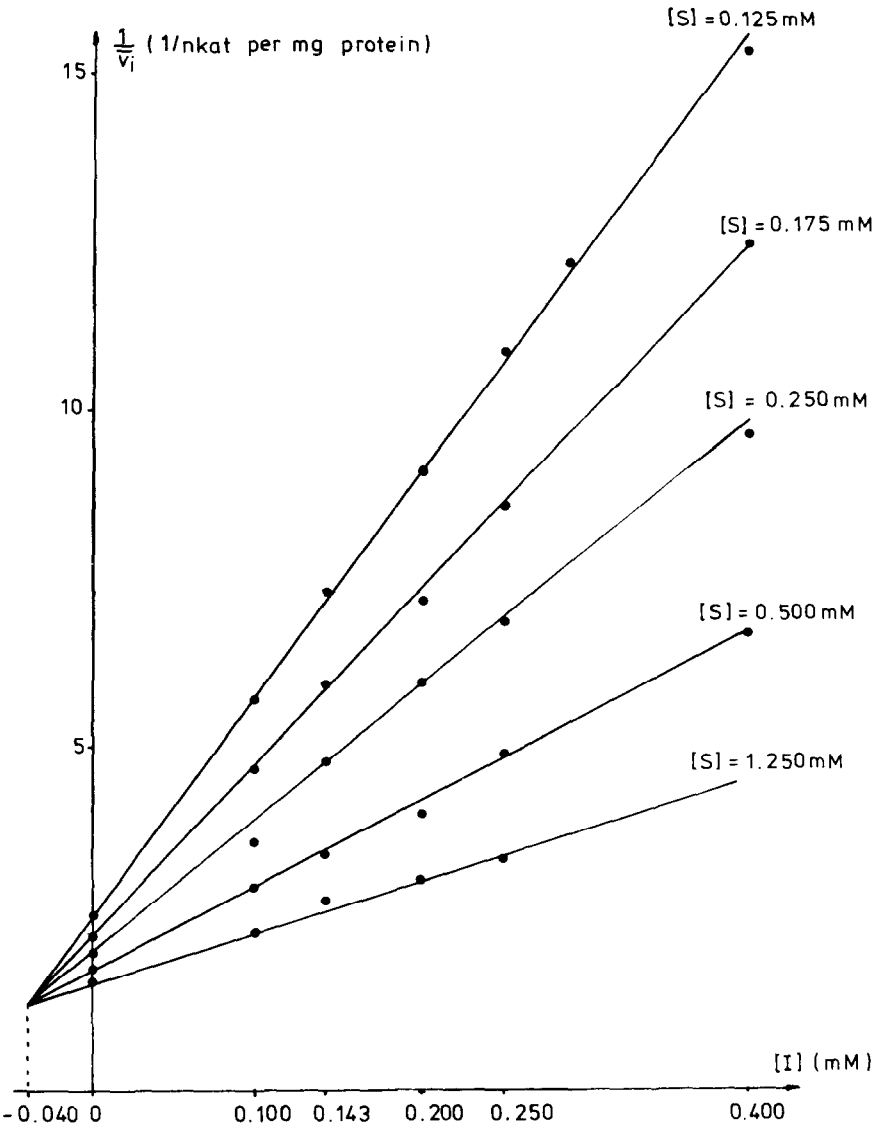

Fig. 4. Determination of the K_i-value of the mixed type inhibition of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction) produced by *N*-feruloyl-Gly-L-PheOH (substrate used: *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H]; radiobiochemical assay) (Dixon plot [12]).

zoxy-L-Phe-L-AlaOH, N^{α}-benzoyl-L-Arg-ethyl ester, *N*-feruloyl-Gly-L-PheOH and *N*-feruloyl-Gly-L-LeuOH*) was investigated.

With most of the latter substrates the usual HPLC assay and the optimal pH for N-FGAH (pH 8), which was not necessarily the optimal pH value of the carboxypeptidases, was employed, while for the determination of the activity of the enzyme with *N*-carbobenzoxy-L-Phe-L-AlaOH a reaction mixture analogous to that used for the *N*-acetylamino acids was employed. For the activity determination with N^{α}-benzoyl-L-Arg-ethyl ester, the increase in extinction of the reaction mixture at 253 nm with time was followed [22, 23] and for testing the possible naphthylamidase activity of the N-FGAH the following substrates were used: L-Leu-*p*-nitroanilide and N^{α}-benzoyl-D,L-Arg-*p*-nitroanilide. With either substrate enzyme activity was followed spectrophotometrically at 410 nm [24].

The possible peptidase activity of N-FGAH was further tested with *N*-L-leucyl-L-tyrosine, *N*-L-alanyl-L-proline, *N*-L-phenylalanylglycine and *N*-L-phenylalanyl-L-alanine as substrates. The reaction mixtures and the assay method used were analogous with those described for the enzymatic splitting of *N*-acetylamino acids.

* The latter two substrates being well suited for the determination of the carboxypeptidase A activity. The pH optimum of carboxypeptidase A with *N*-feruloyl-Gly-L-PheOH was found to be pH 6.8 [21] but the carboxypeptidases isolated by Mikola [19] from barley seeds showed with *N*-carbobenzoxydipeptides, pH optima between pH 5.0 and 5.7.

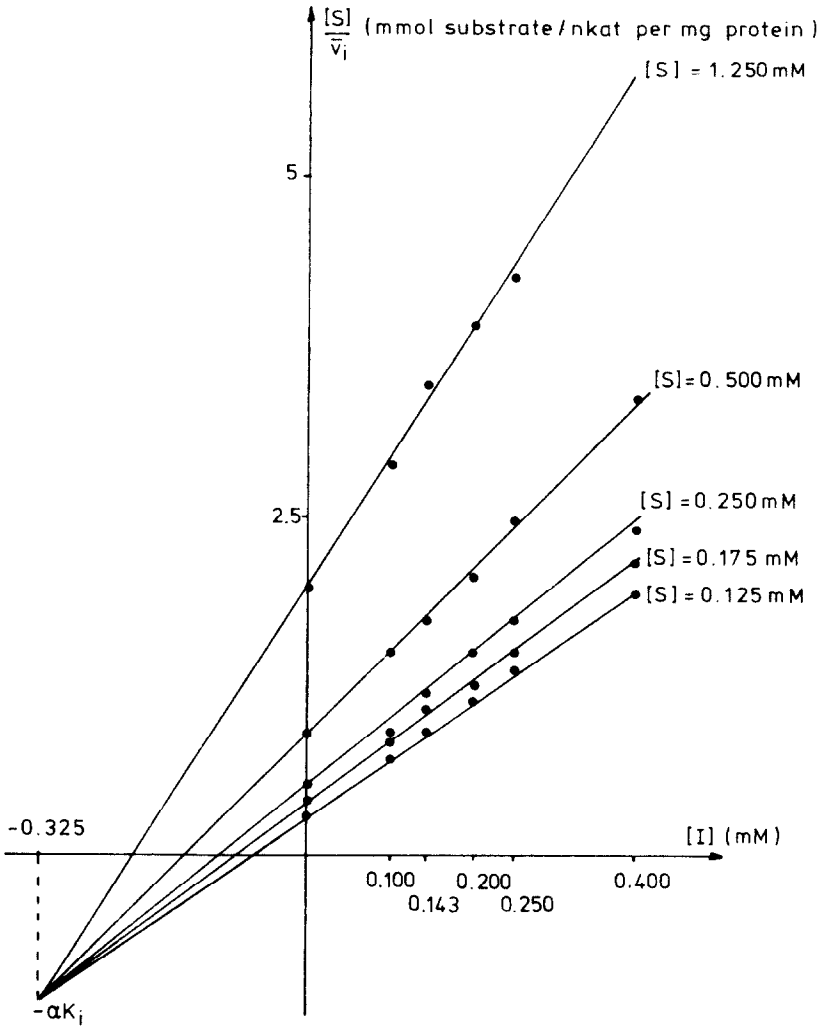

Fig. 5. Determination of the α-value of the mixed type inhibition of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction), produced by *N*-feruloyl-Gly-L-PheOH (substrate used: *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H]; radiobiochemical assay) (Cornish-Bowden plot [13]).

From all the substrates used for testing the possible exo- and endopeptidase activity of the purified N-FGAH none was hydrolysed. In addition, *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] was neither split at 30° (6 hr reaction) by a series of proteolytic enzymes such as: leucine aminopeptidase (EC 3.4.11.1) (hog kidney), peptidase (a preparation with general proteolytic and aminopeptidase activity from hog gut mucosa), carboxypeptidase A (EC 3.4.17.1) (bovine pancreas), carboxypeptidase B (EC 3.4.17.2) (hog pancreas), α-chymotrypsine (EC 3.4.21.1) (bovine pancreas), trypsine (EC 3.4.21.4) (bovine pancreas), subtilisin (EC 3.4.21.14) (*Bacillus subtilis*) and papain (EC 3.4.22.2) (*Carica papaya*; latex).

The new enzyme was furthermore not activated by Co^{2+} ions (1 mM $CoSO_4$ was even inhibitory); the latter ions are well known activators of the *N*-aminoacylase from animal origin [25, 26]. It seems therefore that this enzyme is a new group-specific plant enzyme, which hydrolyses *N*-feruloylglycine with the highest physiological efficiency.

EXPERIMENTAL

Plant material. Barley (*Hordeum vulgare*; cv Zephyr) originating from the test fields of the European barley convention at Proven (West Flanders, Belgium) was used throughout this work. The germination of the seeds and the sterile excision of the embryos were carried out as described [1].

Substrates

Unlabelled compounds. The following substrates: *N*-acetyl-L-methionine, *N*-acetyl-L-alanine, *N*-acetyl-L-leucine, *N*-acetyl-L-aspartic acid, *N*-acetyl-L-tryptophan, N^2-acetyl-L-ornithine, N^6-acetyl-L-lysine, *N*-formyl-L-aspartic acid, *N*-carbobenzoxy-L-phenylalanyl-L-alanine, N^a-benzoyl-L-arginine ethyl ester, L-leucine-*p*-nitroanilide, N^a-benzoyl-D,L-arginine-*p*-nitroanilide, L-alanyl-L-proline, L-phenylalanylglycine, L-phenylalanyl-L-alanine, Azocoll (proteolytic substrate) were purchased from Sigma, U.S.A. *N*-Acetyl-L-phenylalanine was purchased from Janssen Pharmaceutica (Beerse, Belgium) and L-leucyl-L-tyrosine from

Table 2. The K_m, V_m, relative affinity ($1/K_m$) and 'Physiological efficiency' (V_m/K_m) of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction) with different *N*-acylamino acids of the benzoyl and cinnamoyl type (k_m and V_m determined via Lineweaver–Burk analyses [2])

Substrate	K_m (mM)	Relative affinity ($1/K_m$) (M^{-1})	V_m (nmol ferulic acid produced/0.1 mg protein/min)	Physiological efficiency (V_m/K_m)
N-Feruloyl-2-[^{14}C]-glycine-2-[^{3}H]	0.086	11 600	3.95	$45.9.10^3$
N-*p*-Coumaroyl-2-[^{14}C]-glycine-2-[^{3}H]	0.385	2600	6.61	$17.2.10^3$
N-Cinnamoyl-2-[^{14}C]-glycine-2-[^{3}H]	0.426	2350	5.03	$11.8.10^3$
N-Feruloyl-L-alanine*	0.217	4610	1.45	$6.7.10^3$
N-Sinapoylglycine*	0.133	7520	0.55	$4.1.10^3$
N-Dihydroferuloylglycine*	0.833	1200	1.81	$2.2.10^3$
N-*p*-Hydroxybenzoyl-1-[^{14}C]-glycine-2-[^{3}H]	1.180	850	1.40	$1.2.10^3$
N-Benzoyl-1-[^{14}C]-glycine-2-[^{3}H]	2.000	500	1.35	$0.7.10^3$
N-Feruloyl-D,L-aspartic acid*	0.909	1100	0.37	$0.4.10^3$
N-Vanilloyl-1-[^{14}C]-glycine-2-[^{3}H]	1.110	900	0.28	$0.3.10^3$
N-Syringoylglycine*	Only poorly hydrolysed			
N-Galloylglycine*	Hydrolysed, but due to oxidations and other reactions the activity of the enzyme with the corresponding substrates could not be accurately measured.			
N-Protocatechuoylglycine*				
N-Caffeoylglycine*				

*In these cases unlabelled substrates and the HPLC-enzyme assay were employed for the determination of the kinetic constants.

Table 3. The activity of N-FGAH from germinated and excised barley embryos (102-fold purified S_{105} fraction) with substrates normally used for the activity determination of analogous amidohydrolases (amongst them acylases, deformylases and amidases)

Substrate	Amidohydrolase for which each substrate is specific	Activity of the N-FGAH
	Acylases	
N-Acetyl-L-methionine		
N-Acetyl-L-alanine	*N*-acylamino acid amidohydrolase (EC 3.5.1.14)	
N-Acetyl-L-leucine		
N-Acetylglycine-2-[^{14}C]		
N-Acetyl-L-aspartic acid	*N*-Acyl-L-aspartic acid amidohydrolase (EC 3.5.1.15)	The barley enzyme does not split any of the substrates indicated under the conditions described under experimental
N-Acetyl-L-phenylalanine		
N-Acetyl-L-tryptophan	Acylase III (EC 3.5.1.X)	
N^2-Acetyl-L-ornithine	N^2-Acetyl-L-ornithine amidohydrolase (EC 3.5.1.16)	
N^6-Acetyl-L-lysine	N^6-Acetyl-L-lysine amidohydrolase (EC 3.5.1.17)	
N-Acetyl-*β*-alanine	*N*-Acetyl-*β*-alanine amidohydrolase (EC 3.5.1.21)	
N-Benzoyl-1-[^{14}C]-glycine-2-[^{3}H]	*N*-Benzoylamino acid amidohydrolase (EC 3.5.1.32) (hippuricase)	The barley emzyme hydrolyses *N*-benzoyl-1-[^{14}C]-glycine-2-[^{3}H] but with a much lower 'physiological efficiency' as *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] (see Table 2)
	Deformylases	
N-Formyl-L-aspartic acid	*N*-Formyl-L-aspartate amidohydrolase (EC 3.5.1.8)	The barley enzyme does not split any of the substrates indicated under the conditions described under experimental
N-Formyl-L-methionine	*N*-Formyl-L-methionine amidohydrolase (EC 3.5.1.31)	
	N-Feruloylamide amidohydrolase (amidase)	
N-Feruloylamide	(EC 3.5.1.X)	

Table 4. Comparison of the aminoacylases from a few higher plants with the barley embryo N-FGAH

Plant and reference	Plant tissue	Purification factor	pH optimum	Activation by Co^{2+}	M_r	Activation energy (kJ/mol)	Substrate specificity and K_m
Brassica campestris [15]	Seed	150	7.2–8.8*	+	no information available	57.74	Enzyme preparation hydrolyses: a. *N*-acetyl- and *N*-formyl-L-amino acids b. dipeptides Highest affinity for *N*-acetyl-L-methionine, *N*-acetyl-L-tyrosine and *N*-acetyl-L-leucine K_m: 0.1–1 mM*
Parkinsonia aculeata [9, 16]	Seed (embryo and cotyledons)	77	7.0–9.0*	+	79.500	42.70	Enzyme preparation hydrolyses: *N*-acetyl-and *N*-formyl-L-amino acids. Highest affinity for *N*-formyl-L-methionine. K_m: 1–10 mM*
Nicotiana tabacum [17]	Leaf	268	7.0–9.0*	+	160.000	no information available	Enzyme preparation hydrolyses: *N*-acetyl- and *N*-formyl-L-amino acids. Highest affinity for *N*-acetyl-L-arginine, *N*-acetyl-L-methionine, *N*-acetyl-L-cysteine and *N*-formyl-L-methionine. K_m: 1–10 mM*
Hordeum vulgare (and some other plants) Martens *et al.* (this paper)	Seed (embryo and aleurone layer)	102	8.0	10 μM 0.1 μM (no effect) 1 mM (inhibitory (*ca* 37%)	155000	43.50	Enzyme hydrolyses: *N*-acyl-L-amino acids of the cinnamoyl and benzoyl series. (*N*-acetyl-, *N*-formyl-amino acids and *N*-feruloylamide not split). Highest affinity for *N*-feruloylglycine. K_m: 0.085 2 mM*

*Varying with the substrate used.

Table 5. The activity of *N*-acylamino acid amidohydrolase (amino-acylase I) (EC 3.5.1.14) from hog kidney with *N*-acetylglycine-2-[^{14}C] and a series of double labelled *N*-acyl amino acids of the benzoyl and cinnamoyl type (the results are expressed as specific activities in pkat/mg protein)

Substrate	Specific activity (pkat/mg protein)	% Activity of the enzyme (with *N*-acetylglycine-2-[^{14}C] as substrate = 100%)
N-Acetylglycine-2-[^{14}C]	745.83	100.00
N-Benzoyl-1-[^{14}C]-glycine -2-[^{3}H]	70.50	9.45
N-Cinnamoyl-2-[^{14}C]-glycine-2-[^{3}H]	55.00	7.37
N-*p*-Hydroxybenzoyl-1-[^{14}C]-glycine-2-[^{3}H]	2.33	0.31
N-Vanilloyl-1-[^{14}C]-glycine-2-[^{3}H]	2.08	0.28
N-Feruloyl-2-[^{14}C]-glycine-2-[^{3}H]	1.60	0.21
N-Feruloyl-2-[^{14}C]-alanine	1.42	0.19
N-*p*-Coumaroyl-2-[^{14}C]-glycine-2-[^{3}H]	0.42	0.06

Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.; *N*-feruloylamide was donated by Prof. H. Geiger (University of Hohenheim, Stuttgart, F.R.G.). The following compounds: *N*-formyl-L-methionine, and *N*-acetyl-β-alanine were prepared by the method of Sheehan and Yang [27]. *N*-syringoyl-, *N*-cinnamoyl-, *N*-feruloyl-, *N*-dihydroferuloyl- and *N*-sinapoylglycine as well as *N*-feruloyl-L-alanine were synthesized as described in ref. [28]. *N*-Protocatechuoyl-, *N*-galloyl- and *N*-caffeoylglycine were prepared as ref. [29]. *N*-Feruloyl-D,L-aspartic acid was synthesized via the classical acid chloride method (Vande Casteele and Van Sumere, unpublished results). *N*-Feruloylglycyl-L-phenylalanine and *N*-feruloylglycyl-L-leucine [substrates and (or) mixed type inhibitors] were synthesized as refs [30, 31].

Labelled compounds. *N*-Acetylglycine-2-[^{14}C] (specific activity: 13.32 MBq/mmol) was synthesized according to ref. [27]. 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 [32] and the active ester method [28] have been used. All other employed labelled substrates were synthesized by means of the active ester method viz: *N*-benzoyl-1-[^{14}C]-glycine-2-[^{3}H] (specific activity: [^{14}C]: 15.17 MBq/mmol; [^{3}H] 58.09 MBq/mmol); *N*-*p*-hydroxybenzoyl-1-[^{14}C]-glycine-2-[^{3}H] (specific activity [^{14}C]: 9.25 MBq/mmol; [^{3}H]: 2.22 MBq/mmol); *N*-vanilloyl-1-[^{14}C]-glycine-2-[^{3}H] (specific activity [^{14}C]: 11.84 MBq/mmol; [^{3}H]: 25.9 MBq/mmol); *N*-cinnamoyl-2-[^{14}C]-glycine-2-[^{3}H] (specific activity [^{14}C]: 3.33 MBq/mmol; [^{3}H]: 3.7 MBq/mmol), *N*-*p*-coumaroyl-2-[^{14}C]-glycine-2-[^{3}H] (specific activity: [^{14}C]: 11.84 MBq/mmol; [^{3}H]: 22.94 MBq/mmol) and *N*-feruloyl-2-[^{14}C]-L-alanine (specific activity: [^{14}C]: 61.42 MBq/mmol).

Radioactivity counting. Radioactivity was measured as previously described [1].

Preparation of the enzyme extract and purification of the enzyme. *N*-Feruloylglycine amidohydrolase (N-FGAH) was isolated from barley embryos and purified as described [1].

Protein determination. Protein was assayed according to ref. [33].

M_r. The M_r of N-FGAH (155000) from germinated and excised barley embryos was estimated according to ref. [34] using Sepharose 6B and protein M_r markers [e.g.: cyt C (M_r: 13000], α-chymotrypsinogen (25000), ovalbumine (45000), serumalbumine (71000), aldolase (147000), catalase (230000) and ferritine (450000).

TLC. TLC on silica gel–cellulose (1:1; w/w) layers was performed as described earlier (see [1] and Van Sumere *et al.* [35, 36]).

Enzyme assays

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 [35,36] and chromatographed with toluene, ethylformate, formic acid (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. Analogous procedures were employed when other labelled substrates were tested.

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 (0.1M; pH 8). Periodically 100 μl of the above mixture were injected in micro sealed 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. [36]. 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).

Kinetic constants determination. Determination of K_m, V_m, *physiological efficiency and apparent activation energy.* The kinetic constants K_m, V_m and the 'Physiological efficiency' (V_m/K_m) [9, 14] were determined by means of the Lineweaver–Burk [2], and the Eadie–Hofstee methods [3, 4]. Initial velocities were as usual determined in repeated experiments with varying substrate amounts employing the radiobiochemical assay.

The apparent activation energy was determined via the integrated Arrhenius equation and the temperature coefficient [7].

Determination of the substrate specificity of N-FGAH (purification: 102-fold) with the labelled substrates (Table 2). Enzyme activity was measured via the radiobiochemical TLC assay and for the non labelled substrates the HPLC-assay was employed. In both cases the substrate concentrations varied between: 0.125 and 1.25 mM and the enzyme content was 0.21 mg protein/ml.

The K_ms and V_ms were determined as described (see Experimental).

Qualitative estimation of the possible activity of N-FGAH (purification: 102-fold) with substrates which are normally employed for the determination of analogous amidohydrolases (amongst them acylases, deformylases and amidases).

In these cases the same conditions were used as described under 'enzyme assay'. However, the substrates (final concentration: 500 nmol substrate per ml) were those indicated in Table 3. After 0 min and 5 hr enzyme action 200 μl of the reaction mixtures were spotted on cellulose thin layers next to known amounts of the substrate and the corresponding amino acid tests. After development with *n*-BuOH–HOAc–H_2O (4:1:5) the thin layers were dried and then treated with 10% NEt_3 in CH_2Cl_2. Subsequently the thin layers were first sprayed with 0.05% fluorescamine [37, 38] in Me_2CO and finally with the above triethylamine solution (increasing and stabilization of the fluorescence reaction with amino acids). After comparison of the fluorescence of both the amino acid test (10 nmol) and the possibly liberated amino acid moiety of the substrate, the possible enzymatic splitting of the latter could be visually established. It was further also feasible to estimate whether the hydrolysis occurred for more or less than 10%. Since none of the substrates of Table 3 was hydrolysed no further quantitative assay method was required. In the case of *N*-acetylglycine-2-[^{14}C] the radiobiochemical TLC assay method was used. For the determination of the possible amidase activity of the N-FGAH preparation *N*-feruloylamide and the HPLC assay (see above) were employed.

Inhibitor studies

Sulphydryl reagents. To 425 μl 0.1 M Tris–HCl buffer (pH 8.0) containing a certain concentration of inhibitor, 50 μl enzyme solution (protein content: 2.1 mg/ml) was added. The mixture which was off and on stirred was kept for either 0, 30, 60 or 120 min at 30°. After the choosen time interval 25 μl of *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] (5 mM) were added (thorough mixing) and enzyme activity was measured as described (radiobiochemical TLC assay).

Metal chelators. To 400 μl 0.1 M Tris–HCl buffer (pH 8.0) containing 12.5 mM metal chelator, 50 μl enzyme solution (protein content: 2.1 mg/ml) were added. After stirring the mixture was equilibrated at 30° for 10 min. Subsequently 50 μl *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] (5 mM) were added (thorough mixing) and enzyme activity measured (radiobiochemical assay).

Reversible N-feruloylpeptide (N-feruloyl-Gly-L-PheOH or N-feruloyl-Gly-L-LeuOH) inhibitors. Proof of the reversible nature of the inhibitors via Sephadex G25 chromatography of the reaction mixture. Sephadex G25 was suspended at room temp. in 0.1 M Tris–HCl buffer pH 8 containing 10% glycerol and 0.1 M $(NH_4)_2SO_4$ (eluent). Subsequently a 27 × 0.8 cm column was prepared. The reaction mixture (radiobiochemical TLC assay) which contained in addition to the normal ingredients 100 μl of a 5 mM solution of either of the above *N*-feruloyldipeptides was, after equilibration (10 min at 30°), charged onto the column. The elution with the above eluent proceeded, at 5°, with a flow rate of 20 ml/hr. Detection was performed at 280 nm with a 'Uvicord LKB' and 2.5 ml fractions were collected. Since the proteins and the *N*-feruloylpeptides were nicely separated the N-FGAH activity of the protein fractions could be easily determined via the radiobiochemical assay and then the percentage enzyme recovery could be calculated.

Determination of the type of inhibition caused by means of N-feruloyl-Gly-L-PheOH or N-feruloyl-Gly-L-LeuOH. To varying initial concentrations of *N*-feruloyl-2-[^{14}C]-glycine-2-[^{3}H] (0.125, 0.175, 0.250, 0.500 or 1.250 mM, corresponding to either: 25; 35; 50; 100 or 250 μl 5 mM substrate) in respectively 775; 765; 750; 700 and 550 μl 0.1 M Tris–HCl buffer pH 8, 100 μl *N*-feruloyl-Gly-L-PheOH of an appropriate concentration (e.g. 4 mM), in the same buffer, and 100 μl enzyme solution (protein content: 2.1 mg/ml) were added. After mixing, enzyme activity was measured as described (radiobiochemical assay). Analogous experiments in which the final inhibitor concentration was varied, between nil and either 0.4 mM *N*-feruloyl-Gly-L-PheOH or nil and 3.5 mM *N*-feruloyl-Gly-L-LeuOH, were performed in order to determine the initial velocities in the repeated experiments. Subsequently the type of inhibition was determined by Lineweaver–Burk [2] or Hanes [11] plottings.

Determination of the K_i and the α-value ($\beta = 0$) for the N-feruloyl-Gly-L-PheOH and N-feruloyl-Gly-L-LeuOH mixed type inhibitors of N-FGAH. The K_i values for both compounds were determined via Hanes [11] plots or the method of ref. [12]. The α values were obtained via Lineweaver–Burk [2], Hanes [11] or Cornish-Bowden [13] plots.

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