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HYDROLYSIS OF ESTER SUBSTRATES OF TRYPSIN AND CHYMOTRYPSIN BY BARLEY CARBOXYPEPTIDASE

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Abstract—Purified barley carboxypeptidase exhibits high activity against a number of *N*-substituted amino acid esters, which are commonly used as synthetic substrates for mammalian and microbial proteinases. The proteinases of barley, on the contrary, do not hydrolyse these compounds. Because many other plants contain carboxypeptidases closely resembling the barley enzyme, we conclude that synthetic ester substrates should not be used to detect proteinase activity in extracts of higher plants. Plant carboxypeptidases also liberate *C*-terminal tryptophan from α-casein. Therefore, casein also is an unreliable substrate for plant proteinases.

INTRODUCTION

WE HAVE shown earlier that germinating barley grains contain at least three different carboxypeptidases, and isolated one of these enzymes in pure form.[1] The purified enzyme hydrolyses a number of carbobenzoxydipeptides (best substrate Z-Phe-Ala) with an optimum pH of 5·2. It does not act on di- or tripeptides, and it is inactivated by di-isopropylfluorophosphate (DFP). In all these properties the barley enzyme resembles other plant carboxypeptidases, which have been purified from orange peel[2,3] and the leaves of French bean,[4–6] and shown to be present in many other plants.[7] Another of the carboxypeptidases of barley grains has recently been purified by Moeller *et al.*[8]

In this paper we report the rather unexpected finding that purified barley carboxypeptidase[1] exhibits high activity against several *N*-substituted amino acid esters, which are commonly used as synthetic substrates for mammalian and microbial proteinases.

RESULTS

The three proteinase substrates most rapidly broken down by purified barley carboxypeptidase were benzoyl-DL-arginine ethyl ester (BAEE), acetyl-L-phenylalanine ethyl ester (Ac-Phe-EE) and acetyl-DL-phenylalanine *p*-nitrophenyl ester (Ac-Phe-NFE). The pH dependence of the reactions is illustrated in Fig. 1. All the optima are at higher pH (6·2–7·0) than the optimum in the hydrolysis of Z-Phe-Ala (5·2).

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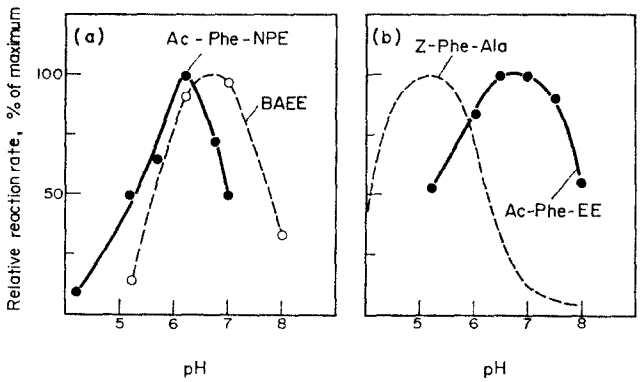

FIG. 1. EFFECT OF pH ON THE HYDROLYSIS OF *N*-SUBSTITUTED AMINO ACID ESTERS BY BARLEY CARBOXYPEPTIDASE.

(a) Hydrolysis of 0·25 mM benzoyl-DL-arginine ethyl ester and 0·083 mM acetyl-DL-phenylalanine *p*-nitrophenyl ester by purified carboxypeptidase.

(b) Hydrolysis of 2 mM acetyl-L-phenylalanine ethyl ester by unfractionated extract of germinating barley, compared to the hydrolysis of 1·82 mM Z-Phe-Ala (redrawn from Ref. 1).

The reaction rates for all the compounds tested are listed in Table 1. The two ester substrates of trypsin (BAEE and BAME) are hydrolysed at rates comparable to the hydrolysis of typical *Z*-dipeptides. The corresponding *p*-nitroanilide (BAPA) and amide (BAA), on the contrary, are not attacked at all. Arginine methyl ester, the unsubstituted ester corresponding to BAME, is not hydrolysed. Apparently, masking of the α-amino group is an essential requirement for the catalytic reaction.

TABLE 1. HYDROLYSIS OF SYNTHETIC SUBSTRATES OF PROTEINASES AND CARBOXYPEPTIDASES BY BARLEY CARBOXYPEPTIDASE

Substrate	v (μmol/min/mg)	c_s (mM)	pH
α-*N*-Benzoyl-DL-arginine ethyl ester (BAEE)	47	0·25	7·0
α-*N*-Benzoyl-L-arginine methyl ester (BAME)	24	0·30	7·5
L-Arginine methyl ester	0	4	5·2–8·4
α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide (BAPA)	0	0·83	8·6
α-*N*-Benzoyl-L-arginine amide (BAA)	0	0·25	5·2–7·5
N-Acetyl-L-phenylalanine ethyl ester (Ac-Phe-EE)	35	2·0	6·8
N-Acetyl-DL-phenylalanine *p*-nitrophenyl ester (Ac-Phe-NPE)	51	0·083	6·2
N-Acetyl-DL-phenylalanine β-naphthyl ester (Ac-Phe-NE)	0·30	0·23	5·2
L-Tyrosine ethyl ester (TEE)	0	2·0	7·9*
Glutaryl-L-phenylalanine-*p*-nitroanilide	0·13	0·83	5·2*
Z-Phe-Ala	118	1·82	5·2
Z-Phe-Ser	13	1·82	5·2
Z-Phe-Phe	9·8	1·82	5·2
Z-Gly-Ala	1·8	1·82	5·2

* Optimum pH for unfractionated extract.

Among the substrates of chymotrypsin, the ethyl and *p*-nitrophenyl esters of acetyl-DL-phenylalanine are rapidly hydrolysed. The corresponding β-naphthyl ester is hydrolysed much more slowly. Unfractionated barley extracts, however, contain two enzymes with relatively high activity on this substrate;[9] these enzymes could of course be the other two carboxypeptidases. A very slow reaction was also observed using glutaryl-L-phenylalanine-*p*-nitroanilide as substrate. The unsubstituted ester, TEE, was not hydrolysed.

The effect of substrate concentration on reaction rate was studied with some of the substrates. The plots of $1/v$ against $1/S$ were linear, and the following K_m values were obtained: BAEE 0·31 mM (pH 7·0), BAME 0·30 mM (pH 7·0), Ac-Phe-NFE 0·094 mM (pH 6·2). The values are much lower than the corresponding figure for Z-Phe-Ala (6·7 mM, pH 5·2).

All barley carboxypeptidases are inactivated by DFP.[10] In order to see whether germinating barley contains other enzymes active on the substituted amino acid esters, some inactivation experiments were carried out with DFP. Extracts of germinating barley hydrolysed all the substrates included in Table 1, except for arginine methyl ester. Treatment of the extracts with 1 mM DFP (1 hr, 25°, pH 5·4) did not have any effect on the hydrolysis of BAPA, BAA, and TEE; the reaction rates for BAEE and BAME were reduced by 84 and 80% respectively, while complete inhibitions were obtained for all the other substrates. Consequently, it seems that all the chymotrypsin substrates, with the exception of TEE, are hydrolysed exclusively by the carboxypeptidases; for BAEE and BAME about 16 and 20% of the total reactions are contributed by other enzymes.[11]

DISCUSSION

The results show definitely that none of the common ester substrates of mammalian and bacterial proteinases can be used to detect proteinase activity in barley extracts. The carboxypeptidases hydrolyse these compounds quite rapidly, while none of the several proteinases of barley[12] seems to hydrolyse these compounds with significant velocity. As mentioned in the introduction, carboxypeptidases have been shown to be present in many higher plants, and all these enzymes closely resemble each other. Therefore, the above considerations probably hold for many other plant extracts as well. However, if there is uncertainty whether a substrate is hydrolysed by a proteinase or a carboxypeptidase, the matter can be settled by use of the selective carboxypeptidase inhibitor, DFP.

It should be noted that use of casein, another standard substrate for proteinases, is equally unreliable. The plant carboxypeptidases liberate *C*-terminal tryptophan from α-casein (the main component in commercial casein preparations). This has been indicated by several studies and most definitely demonstrated for the other carboxypeptidase of barley by Moeller *et al.*[8] This single tryptophan residue accounts for a considerable part of the total UV absorption of casein solutions. Therefore, if casein is used to measure proteinase activity of plant extracts, and activity is equated with the production of TCA-soluble UV absorbing peptides, up to 50% of the apparent proteinase activity can be due to tryptophan liberated by the carboxypeptidases.[13]

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Burger *et al.* have earlier studied a barley peptidase active on BAEE[11] (BAEEase, barley peptide hydrolase *B*). The very high activity of our carboxypeptidase on BAEE as well as similar pH optima and K_m values (0·31 mM vs. 0·38 mM) show that the two enzymes are identical. However, our pure enzyme preparations, in contrast to the partially purified preparations of Burger *et al.*, did not hydrolyse di- or tripeptides. We have confirmed our earlier results using L-glutamyl-L-asparagine, one of the two peptides most rapidly hydrolysed in Burger's experiments. After incubation with high concentrations of purified carboxypeptidase the reaction products were analyzed with an amino acid analyzer; no hydrolysis was observed.

Barley carboxypeptidase resembles trypsin and chymotrypsin and also the microbial 'serine' proteinases in its sensitivity to DFP and in its ability to hydrolyse *N*-substituted amino acid esters. These similarities probably reflect some analogies in the respective reaction mechanisms. In the case of the French bean carboxypeptidase Shaw and Wells have actually shown that DFP inactivates the enzyme by reacting with a serine residue.[6] The amino acid sequence around the 'active' serine (-Glu-Ser-Val-), however, differs from the corresponding sequences of the other 'serine' proteinases.

EXPERIMENTAL

Enzyme. The preparation of barley carboxypeptidase was a part of the batch described in Ref. 1. It had been prepared from Pirkka barley (a Finnish 6-row cultivar). The enzyme had been stored for 2 yr as a precipitate suspension in 60% $(NH_4)_2SO_4$ soln. (pH 5·4) at +5°. The activity had decreased during the storage from 180 to 118 U/mg. Extracts of germinated barley were prepared as described earlier;[1] the small-molecular compounds were removed by gel filtration in a column of Sephadex G25 equilibrated with 10 mM NaCl, and the large-molecular fraction was stored in small aliquots at −18°.

Reagents. The enzyme substrates were obtained commercially.

Methods. Spectrophotometric methods were used to follow the hydrolysis of Ac-Phe-NPE (349 nm),[14] BAEE, BAME and BAA (252 nm),[11] and BAPA and glutaryl-L-phenylalanine-*p*-nitroanilide (410 nm).[15] The hydrolysis of Ac-Phe-NE was estimated by coupling the liberated β-naphthol with Diazo Blue B.[16] The hydrolysis of Ac-Phe-EE and TEE was followed titrimetrically; the liberated carboxyl groups were continuously titrated with 10 mM NaOH using an automatic titrator. Carboxypeptidase activities were determined with our earlier method[1] (a more convenient and more accurate method has been developed later[17]). In all enzyme assays NaOAc, sodium phosphate, and Tris-HCl buffers were used in the pH ranges 5–6, 6–7·5 and 7·5–8·5 respectively. The buffer concentrations were 50 mM except in the titrimetric assays, where 5 mM buffers were employed. All the enzymic reactions were carried out at 30°, except for the titrations which were performed at room temp.

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