Biochimica et Biophysica Acta, 350 (1974) 495—498

BBA Report

BBA 61292

THE ACTIVE SITE OF PORCINE ENTEROPEPTIDASE

SELECTIVE INACTIVATION OF THE PEPTIDASE ACTIVITY

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(Received April 9th, 1974)

Summary

Porcine enteropeptidase has been shown to have an extended active site comprising two subsites: a heat-stable catalytic site where bond hydrolysis occurs and a heat-labile specificity site which recognises the $(Asp)_4$ sequence of trypsinogen. If the specificity site is destroyed then loss of activity towards trypsinogen occurs even though the catalytic site remains intact.

Enteropeptidase (EC 3.4.21.9) activates bovine trypsinogen by cleaving the same Lys—Ile bond (between residues 6 and 7) as does trypsin [1—3]. Immediately adjacent to this Lys—Ile sequence is a cluster of four aspartyl residues (Residues 2 to 5) and this $(Asp)_4$ sequence is common to all trypsinogens so far sequenced with the one exception, that obtained from the African lungfish, where the corresponding sequence is Ile—$(Glu)_2$—Asp—Lys (Residues 3 to 7) [4].

Using synthetic peptides Maroux et al. [5] showed that porcine enteropeptidase recognises not only the basic residue of the Lys—Ile bond cleaved during the activation process but also the adjacent cluster of acidic residues. However, enteropeptidase will also hydrolyse benzoyl-L-arginine ethyl ester (BAEE) and tosyl-L-arginine methyl ester both of which lack the cluster of acidic residues. Therefore further attempts have been undertaken to determine the significance of the polyaspartyl sequence of trypsinogen. In this report it is shown that the site in porcine enteropeptidase which recognises and binds the polyaspartyl cluster is heat-labile and that

Abbreviation: BAEE, benzoyl-L-arginine ethyl ester.

productive binding of trypsinogen to enteropeptidase is dependent on this site being intact.

The peptidase activity of enteropeptidase was determined as previously described using bovine trypsinogen as substrate [6]. The esterase activity of enteropeptidase was estimated at 30 °C with BAEE as substrate. The assay mixtures (4.2 ml) contained 0.1 mM dimethylglutarate (Na^+), pH 5.8, 2mM NaCl or $CaCl_2$ and 1.45 mM BAEE. The reaction was initiated by the addition of 0.1 ml enteropeptidase and the pH was maintained at 5.8 with 2 mM NaOH using a Radiometer Type TTTld pH Stat. One esterase unit of enteropeptidase activity catalyses the hydrolysis of 1 μmole of BAEE per min. Under the conditions described here, the rate of NaOH uptake was proportional to the amount of enteropeptidase added up to at least 0.088 unit of enzyme.

The peptidase activity of enteropeptidase is heat labile and Ca^{2+} has been shown to stabilise this activity against heat denaturation [6]. However when enteropeptidase was incubated for short periods and the residual esterase activity determined with BAEE, the enzyme showed no heat lability compared with an 84% loss of the activity towards trypsinogen, i.e. the peptidase activity of enteropeptidase had been selectively destroyed (Table I).

TABLE I

COMPARISON OF THE HEAT STABILITY OF THE PEPTIDASE AND ESTERASE ACTIVITIES OF ENTEROPEPTIDASE

Enteropeptidase was incubated at 62 °C in a solution (0.2ml) containing 0.122 peptidase units of enteropeptidase, 10.0 mM dimethylglutarate (Na^+), pH 5.8, and 4 mM $CaCl_2$ (if present). After 7 min, the solutions were rapidly cooled in an ice bath and the residual peptidase and esterase activities were determined.

Additions	% Residual Peptidase Activity	% Residual Esterase Activity
4 mM $CaCl_2$	100	100
No additions	15.7	100

Since hydrolysis of BAEE is not readily destroyed by heat, the catalytic site must remain intact. It must be this which recognises the essential basic amino acid residue of the substrates and which catalyses the hydrolysis of the arginine-ethyl ester bond of BAEE and the Lys—Ile bond of trypsinogen. The region of the enteropeptidase molecule which is heat-labile must be that part of the active site which is specifically required for the activation of trypsinogen. Based on the specificity requirements of enteropeptidase [5] it is reasonable to conclude that this heat-labile region is a recognition or specificity site for the polyaspartyl sequence of trypsinogen.

This conclusion was supported by the following observations. Firstly, the K_m for trypsinogen increased as heat denaturation progressed (Fig. 1). After 5 min, 30% of the peptidase activity was lost and the K_m had increased 3-fold to 0.1 mM and after 12 min, the K_m was 0.3 mM and 85% of the activity was destroyed. Secondly, the dipeptide, Glu—Asp (a model for the

TABLE II

THE PROTECTION OF ENTEROPEPTIDASE AGAINST HEAT DENATURATION BY GLUTAMYL-ASPARTATE

Enteropeptidase was preincubated at 61 °C in a solution (0.2ml) containing 0.011 peptidase units of enteropeptidase and 10 mM dimethylglutarate (Na^+), pH 5.8, in addition to the components listed above. After 8.5 min, the solution was rapidly cooled and the residual peptidase activity was determined.

Additions	Concentration (mM)	% Residual activity
Control		
no incubation		100
No additions		19.8
Ca^{2+}	0.05	79.5
Glu-asp	12	36.8
Glu-asp	5	32.6

polyaspartyl sequence) partially protected the heat-labile site although it was much less effective than Ca^{2+} (Table II). Finally, trypsinogen where an average of two carboxyl groups of the activation peptide had been selectively but randomly modified by glycinamide [7,8] was a poorer substrate for enteropeptidase than was native trypsinogen since the K_m for trypsinogen increased two-fold from 0.021 mM to 0.04 mM following modification with no change in V.

These observations show that enteropeptidase has an extended active site and confirm the presence of a region in the active site of enteropeptidase which specifically recognises and binds the polyaspartyl sequence of trypsinogen. This site is heat-labile, is readily stabilised by low levels of $Ca^{2+}$and

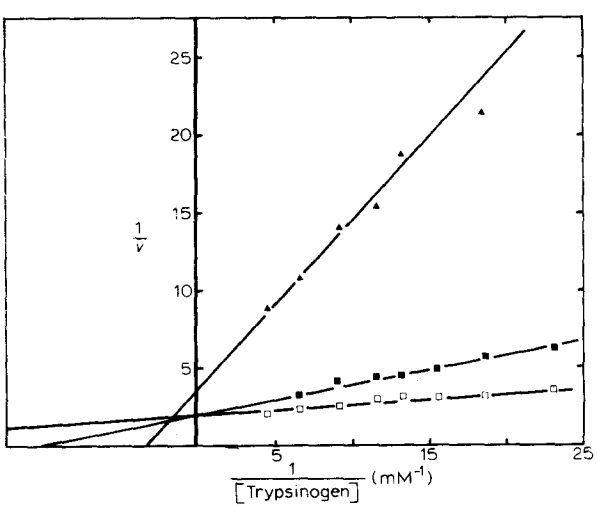

Fig.1. Double-reciprocal plots of the initial velocity of the peptidase activity against trypsinogen concentration following preincubation of enteropeptidase at 60 °C for 0 min (□—□), 5 min (■—■) and 12 min (▲—▲). Preincubation was carried out in a solution (0.5 ml) containing 0.055 peptidase units of enteropeptidase and 10 mM dimethylglutarate (Na^+), pH 5.8. Aliquots (50 μl) of the preincubated enzyme were assayed for 10 min for peptidase activity in solutions (0.2 ml) containing 0.1M dimethylglutarate (Na^+), pH 5.8, 3 mM Ca^{2+} and varying trypsinogen concentrations.

is topographically separate from the heat-stable catalytic site where bond hydrolyses occur. The responsibility for the recognition of the essential basic residue of enteropeptidase substrates must reside in the catalytic site although productive binding of trypsinogen can only occur when the polyaspartyl sequence of the activation peptide of the substrate is recognised and bound by the intact specificity site.

The $(Asp)_4$ sequence of trypsinogen appears to play an important role in the binding of trypsinogen to enteropeptidase. If this sequence is modified or if that site on the enzyme is destroyed where the $(Asp)_4$ sequence is bound, then loss of activity results even though the catalytic site itself remains intact.

Acknowledgements

This work was supported in part by the National Health and Medical Research Council, the Clive and Vera Ramaciotti Foundations and The Queen Elizabeth Hospital Medical Research Foundation.

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