Biochimica et Biophysica Acta, 321 (1973) 624–631

BBA 67013

THE EFFECTS OF Ca^{2+} ON PORCINE ENTEROPEPTIDASE ACTIVITY

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(Received March 19th, 1973)

SUMMARY

The activity of porcine enteropeptidase was inhibited by low concentrations of EDTA suggesting a requirement for a divalent metal ion in the catalysis. The Group IIA metal ions, Ca^{2+}, Sr^{2+} and Ba^{2+}, were found to stimulate enzymic activity in the order, $Ca^{2+} > Sr^{2+} > Ba^{2+}$. Stimulation by Ca^{2+} at pH 5.8 was achieved by a 2-fold decrease in the K_m for trypsinogen with essentially no change in V. Ca^{2+} was also found to have a marked stabilising effect when the enzyme was subjected to thermal denaturation. The pH dependence of these two effects of Ca^{2+} was markedly different. The stimulation of enzymic activity showed a maximum around pH 5.0 and a minimum about pH 6.0, whereas the stabilisation against heat treatment showed a maximum around pH 6.0 and a minimum below pH 5.0.

INTRODUCTION

Enteropeptidase (previously referred to as enterokinase, EC 3.4.4.8) is a proteolytic enzyme found in duodenal fluid[1], and in association with the epithelial lining of the upper small intestine[2]. Its substrate, trypsinogen, enters the duodenum in the pancreatic secretion where it is converted by enteropeptidase to trypsin which, in turn, activates the other pancreatic proenzymes, *viz.* chymotrypsin, procarboxypeptidase, proelastase and prophospholipase. Although trypsin will readily activate trypsinogen *in vitro* in mildly alkaline conditions[3], the *in vivo* conditions are such that this action of trypsin is severely limited. Consequently, a deficiency of enteropeptidase results in marked disruptions to protein digestion[4].

Maroux *et al.*[5] showed that both porcine enteropeptidase and trypsin are inactivated by the active-site-directed irreversible inhibitors, tosyllysine chloromethylketone and diisopropylphosphofluoridate, and that both hydrolyse the synthetic substrates, benzoyl-L-arginine ethyl ester and tosyl-L-arginine methyl ester. Consequently, the catalytic sites of the two enzymes must be similar.

These workers were unable to show any effect of Ca^{2+} on the kinetic parameters of enteropeptidase activation of trypsinogen whereas the involvement of this metal ion in the tryptic activation of trypsinogen is well established[6–8]. In this report, we

show that enteropeptidase activation of trypsinogen is influenced by the presence of added Ca^{2+} ions. These ions both stimulate enteropeptidase activity and protect the enzyme against heat denaturation.

MATERIALS AND METHODS

N-α-Benzoyl-L-arginine-*p*-nitroanilide was a product of either Sigma or Schwarz-Mann. Bovine trypsinogen and calcium acetate were purchased from Sigma. All other reagents were analytical grade where possible.

A trypsinogen solution (25 mg/ml, 5 mM HCl) was dialysed against 1 mM HCl at 4 °C and then centrifuged to remove insoluble material. The protein concentration was adjusted to 10 mg/ml ($A_{1cm}^{1mg/ml} = 1.5$ at 280 nm (ref. 9)) and the solution stored frozen.

Estimation of enteropeptidase activity

Enteropeptidase was assayed by the activation of trypsinogen. Assay mixtures (total volume 0.4 ml) contained (in μmoles): 3,3'-dimethylglutarate (sodium salt) (pH 5.8), 20; calcium acetate, 1.2; trypsinogen, 1.0 mg. The reaction was initiated with enteropeptidase and stopped by the addition of 100 μl 0.5 M HCl. The incubation was at 30 °C.

The trypsin liberated during this incubation was estimated on a 20-μl aliquot using the method of Erlanger *et al.*[10]. A stock solution of *N*-α-benzoyl-L-arginine-*p*-nitroanilide was made by dissolving 43.5 mg in 1 ml dimethylsulphoxide, and the substrate solution was prepared by adding 10-μl aliquots of this stock solution to 1 ml of 0.1 M Tris-HCl (pH 8.2), containing 0.02 M calcium acetate, previously warmed to 30 °C (care being taken to ensure that the temperature of this substrate solution does not fall below 25 °C). The trypsin was determined spectrophotometrically at 30 °C by measuring the rate of increase of absorbance at 410 nm.

One unit of trypsin catalyses the hydrolysis of 1 μmole *N*-α-benzoyl-L-arginine-*p*-nitroanilide per min under the conditions of the assay; one unit of enteropeptidase will liberate one unit of trypsin from trypsinogen per min under the specified conditions.

The trypsinogen concentration used in this procedure is up to two orders of magnitude higher than that used by other workers[2,5,11–14]. Consequently, the sensitivity of this method is considerably increased and the observed activity is much less dependent on the purity of the trypsinogen since the concentration of this substrate is about 3-fold higher than its K_m. Despite this high trypsinogen concentration, autoactivation is not kinetically significant as shown in Fig. 1. Under the conditions described here, trypsin formation is proportional to the enteropeptidase added at the time of incubation at least up to 0.05 units of enteropeptidase during a 20-min assay period.

Preparation of Ca^{2+}-free enteropeptidase

Porcine enteropeptidase was prepared from duodenal fluid obtained from the abatoirs immediately following the death of the animals. The purification procedure used was an unpublished method developed in this laboratory involving autolysis[15], $(NH_4)_2SO_4$ fractionation, Sephadex G-200 gel filtration and DEAE-Sephadex ion-

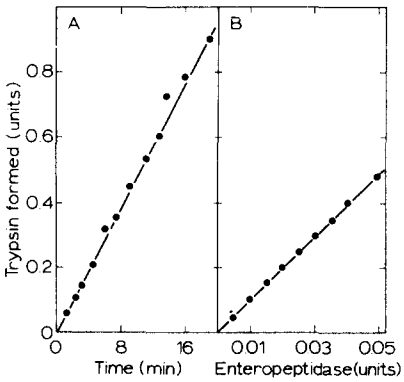

Fig. 1. The determination of enteropeptidase activity with bovine trypsinogen as substrate. Assay solutions (0.4 ml) contained (in μmoles): 3,3'-dimethylglutarate (sodium salt) (pH 5.8), 20; calcium acetate, 1.2; trypsinogen, 1.0 mg. Incubation was at 30 °C and the reaction was stopped with 0.1 ml 0.5 M HCl and the trypsin formed was determined on a 20-μl aliquot by the method of Erlanger *et al.*[10] as described in Materials and Methods. A. Enteropeptidase (0.048 units) was incubated with trypsinogen for varying times. B. Varying amounts of enteropeptidase (up to 0.047 units) were incubated for 10 min.

exchange chromatography. Following autolysis, no free tryptic activity was detected.

The purified enzyme was made Ca^{2+}-free using a Sartorius collodion vacuum dialysis bag. The volume of the enteropeptidase solution was brought to a minimum with concomittant dialysis against 0.1 mM EDTA following three successive additions of 0.5 ml distilled water. The enzyme was then diluted to the appropriate volume with distilled water. Without this treatment, the degree of stimulation by Ca^{2+} was variable since some loosely bound Ca^{2+} was always carried through the purification procedure.

RESULTS

The activity of porcine enteropeptidase towards trypsinogen was found to be sensitive to low levels of EDTA suggesting that the enzyme required the presence of a divalent metal ion for the full expression of activity (Table I). The Group IIA metal

TABLE I

INHIBITION OF ENTEROPEPTIDASE ACTIVITY BY EDTA

Enteropeptidase was preincubated for 30 min at 30 °C in a solution (final volume 0.22 ml) containing 0.045 units of enzyme, EDTA as indicated and 18 mM 3,3'-dimethylglutarate (sodium salt) (pH 5.8). Aliquots (50-μl) were withdrawn and assayed for residual activity as described in Materials and Methods.

EDTA concn (mM)	*% residual activity*
10	66
1	70
0.3	74
0.1	87
0.03	96
0.01	100
Control	100

ions, Ca^{2+}, Sr^{2+} and Ba^{2+}, were found to stimulate enteropeptidase activity (Table II). Ca^{2+} was the most active producing a 25% increase in activity when present at 5 mM although beyond that level inhibition occurred. Ba^{2+} showed only a slight stimulation while the effect of Sr^{2+} was intermediate between the two. Rat enteropeptidase is also readily inhibited by EDTA although stimulation of enzymic activity by Ca^{2+} was not shown by the crude enzyme preparations used[16].

TABLE II

EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY OF ENTEROPEPTIDASE

The assay solutions (0.4 ml) containing (in μmoles): 3,3'-dimethylglutarate (sodium salt) (pH 5.8), 20; divalent metal ions as indicated, 2.0; trypsinogen, 1.0 mg. The reaction was initiated with 0.007 units of enteropeptidase, incubated for 10 min at 30 °C and then processed as described in Materials and Methods.

Metal	*% activity*
No metal	100
Ba^{2+}	105
Ca^{2+}	125
Co^{2+}	92
Mn^{2+}	78
Mg^{2+}	95
Sr^{2+}	113
Zn^{2+}	78

The stimulation by Ca^{2+} was found to be pH dependent with the greatest degree of activation around pH 5.0 and decreasing to a minimum around pH 6.0 (Fig. 2). Above pH 6.0, Ca^{2+} also markedly stimulated trypsin formation although, in this region, tryptic activation of trypsinogen begins to be significant (Fig. 3). Therefore, analysis of the Ca^{2+} stimulation of the enteropeptidase activity above pH 6.0 is not possible with trypsinogen as substrate since two simultaneous reactions are occurring both of which are sensitive to added Ca^{2+}.

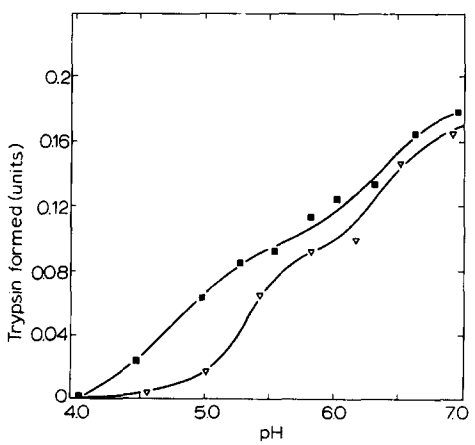

Fig. 2. The relationship between pH and enteropeptidase activity was determined in the presence (■—■) and absence (△—△) of 5 mM Ca^{2+}. Assay mixtures (0.4 ml) contained (in μmoles); 3,3'-dimethylglutarate (sodium salt) (pH as indicated), 20; calcium acetate (if present), 2.0; trypsinogen, 1.0 mg. The reaction was initiated with 0.011 units of enteropeptidase, incubated for 10 min at 30 °C and then processed as described for Fig. 1.

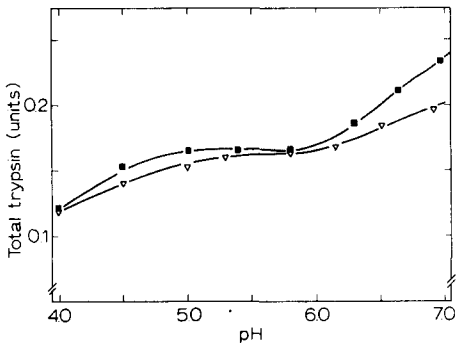

Fig. 3. The relationship between pH and tryptic activation of trypsinogen was determined in the presence (■—■) and absence (△—△) of 5 mM Ca^{2+}. Conditions were as in Fig. 2 except that 0.12 units of trypsin dialysed against 1 mM HCl replaced the enteropeptidase.

In the case of the tryptic activation of trypsinogen at pH 8.0, the stimulation induced by added Ca^{2+} is achieved by a 3-fold decrease in the K_m for trypsinogen with no significant change in the maximum velocity of the reaction[8]. An analogous situation was found with the enteropeptidase catalysed activation of trypsinogen at pH 5.8. The stimulation produced by added Ca^{2+} is predominantly the result of a 2-fold decrease in the apparent K_m for trypsinogen from 0.066 to 0.034 mM (Fig. 4). In fact the value for the K_m of trypsinogen in the absence of Ca^{2+} (0.066 mM) is comparable to that obtained by Maroux *et al.*[5] (0.07 mM).

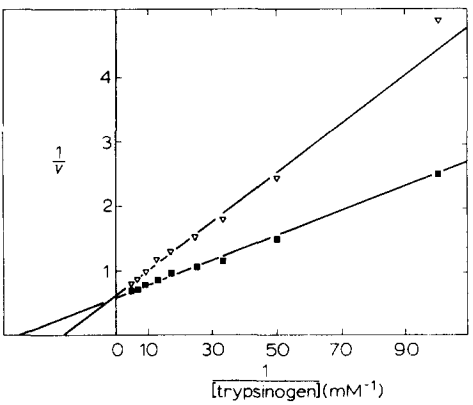

Fig. 4. Double-reciprocal plots of the initial velocity of enteropeptidase activity against trypsinogen concentration obtained in the presence (■—■) and absence (△—△) of 5 mM Ca^{2+}. Assay mixtures (0.4 ml) contained (in μmoles): 3,3-dimethylglutarate (sodium salt), (pH 5.8), 20; calcium acetate (if present), 2.0; trypsinogen as indicated. The reaction was initiated with 0.021 units of enteropeptidase, incubated at 30 °C for 10 min and then processed as described for Fig. 1.

By way of contrast, Ca^{2+} has essentially no effect on the tryptic activation of trypsinogen under conditions similar to that used for the activation by enteropeptidase (Fig. 5).

Porcine enteropeptidase undergoes rapid thermal denaturation when incubated at 60 °C (ref. 14). It was found, however, that if Ca^{2+} was present during the incu-

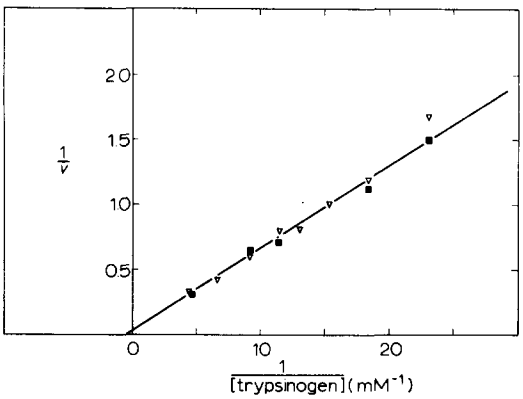

Fig. 5. Double-reciprocal plots of the initial velocity of tryptic activation of trypsinogen against trypsinogen concentration obtained in the presence (■—■) and absence (△—△) of 5 mM Ca^{2+}. Conditions were as described for Fig. 4 except that 0.18 units of trypsin dialysed against 1 mM HCl were used instead of enteropeptidase.

bation enteropeptidase was completely stabilised over short periods. Of all the metal ions tested at the 5-mM level, Ca^{2+} alone was effective in this respect (Table III). This protection afforded by Ca^{2+} was also pH dependent showing a maximum at pH 5.5–6.5 and a minimum below pH 5.0 (Fig. 6). Again, an analogy can be drawn between this observation and the stabilisation of trypsin by Ca^{2+} (refs 6 and 7).

It could be argued that, despite the remarkable resistance of enteropeptidase to proteolytic attack[15], the autolysis to which the enzyme used in this study was subjected could have caused it to become sensitive to the presence of Ca^{2+}. However, this appears unlikely since enteropeptidase prepared from non-autolysed duodenal fluid shows similar responses to Ca^{2+}, *viz.* EDTA inhibition, stimulation by Ca^{2+} and protection against heat denaturation by these metal ions. Therefore, previous failures to detect the divalent metal ion requirement of enteropeptidase would result from the fact that steps had not been taken to free the enzyme of the Ca^{2+} carried through the purification procedure.

TABLE III

EFFECT OF DIVALENT METAL IONS ON THE HEAT STABILITY OF ENTEROPEPTIDASE

The enzyme was incubated at 62 °C for 7.5 min in a solution (final volume 0.2 ml) containing 0.022 units of enteropeptidase, 20 mM 3,3′-dimethylglutarate (sodium salt) (pH 5.8), and various divalent metal ions at the 5 mM level. The solutions were rapidly cooled and residual activity was assessed on 100-μl aliquots as described in Materials and Methods.

Metal	*% residual activity*
Control (no incubation)	100
No metal	5
Ba^{2+}	5
Ca^{2+}	112
Co^{2+}	7
Cu^{2+}	13
Mg^{2+}	5
Mn^{2+}	5
Zn^{2+}	5

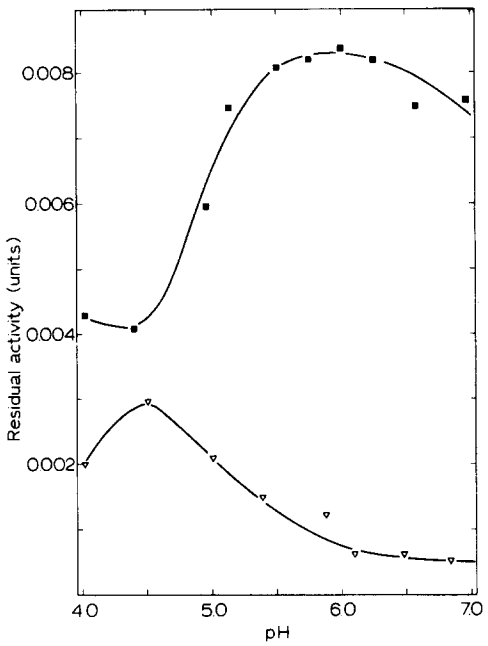

Fig. 6. The enzyme was incubated at 60 °C for 7 min in a solution (final volume 0.12 ml) containing 0.0084 units of enteropeptidase 34 mM 3,3′-dimethylglutarate (sodium salt) (pH 4.0–7.0), and 1.7 mM Ca^{2+} (if present). The solutions were rapidly cooled and the residual activity was assessed on 50-μl aliquots as described in Materials and Methods. Residual activity in the presence (■—■) and absence (△—△) of Ca^{2+} is plotted against the pH.

DISCUSSION

Maroux *et al.*[5] reported that trypsin and enteropeptidase shared many specificity properties especially with regard to their behaviour towards active-site directed irreversible inhibitors and synthetic trypsin substrates. The results reported here show that these similarities can now be extended to include their response to added Ca^{2+}.

In the activation of trypsinogen by trypsin at pH 8.0, Ca^{2+} has a dual function. Ca^{2+} binds to the trypsin moiety of trypsinogen and when in this position they ensure a minimum formation of inert protein[6,7] by inducing a conformational change in the protein[8]. The second binding site of Ca^{2+} is the two aspartyl residues (Residues 4 and 5 in bovine trypsinogen) immediately adjacent to the susceptible Lys–Ile bond (Residues 6 and 7) hydrolysed during the activation process. The binding of Ca^{2+} to this site results in a 3-fold decrease in the K_m for trypsinogen[17]. This effect would appear to be achieved by neutralising the high negative charge associated with the polyaspartyl sequence[18].

The means whereby Ca^{2+} produces a decrease in the K_m for trypsinogen with enteropeptidase is as yet unknown. A simple role of charge neutralisation of the polyaspartyl region of trypsinogen, as appears to be the case with the tryptic activation[18], could not apply to enteropeptidase since Ca^{2+} also influences the rate of hydrolysis of benzoyl arginine ethyl ester by porcine and human enteropeptidase and

this substrate does not possess the necessary polyaspartyllysine structure (Barns, R. J. and Elmslie, R. G., unpublished).

Like trypsin, the requirement for divalent metal ions does not appear to be absolute since only 30–40% of the initial enteropeptidase could be destroyed by EDTA (Table I). However, the marked difference in the pH optimum for the stimulation of enteropeptidase activity by Ca^{2+} (about pH 5.0) and the Ca^{2+}-induced protection of enteropeptidase against thermal denaturation (pH 5.5–6.5) may be significant in understanding the role of Ca^{2+}. A dual function of Ca^{2+} could explain this difference.

ACKNOWLEDGEMENT

This work was supported in part by The Queen Elizabeth Hospital Medical Research Foundation.

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