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HYDROLYSIS OF ARTIFICIAL SUBSTRATES BY ENTEROKINASE AND TRYPSIN AND THE DEVELOPMENT OF A SENSITIVE SPECIFIC ASSAY FOR ENTEROKINASE IN SERUM

D.A.W. GRANT and J. HERMON-TAYLOR

Department of Surgery, St. George's Hospital Medical School, Cranmer Terrace, London, SW17 ORE (U.K.)

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Summary

The activities of highly purified human enterokinase (enteropeptidase, EC 3.4.21.9) and bovine trypsin were tested against three synthetic substrates α-*N*-Benzoyl-L-arginine ethyl ester HCl, α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide HCl and α-*N*-Benzoyl-DL-arginine-2-naphthylamide HCl. There was no detectable hydrolysis of these substrates by enterokinase whereas the kinetic parameters obtained for trypsin were in close agreement with those previously described by other workers. The values for K_m and k_{cat} were dependent on the Ca^{2+} concentration. Hydrolysis of glycine-tetra-L-aspartyl-L-lysyl-2-naphthylamide (Gly-$(Asp)_4$-Lys-Nap) by these proteases was also studied. Enterokinase-catalysed hydrolysis obeyed simple steady-state kinetics and values for K_m of 0.525 mM and 0.28 mM and for k_{cat} of 21.5 s^{-1} and 28.3 s^{-1} were obtained in 0.1 mM and 10 mM Ca^{2+}, respectively. Trypsin-catalysed hydrolysis was complex and the response to Ca^{2+} was sigmoidal partly due to the lability of trypsin at low Ca^{2+} concentrations. A sensitive specific assay for enterokinase was developed and applied to the measurement of the enzyme in serum; interference by non-specific arylamidases was eliminated by the addition of Zn^{2+}.

Introduction

The usual method for assaying enterokinase (enteropeptidase, EC 3.4.21.9) is by activation of its normal biological substrate trypsinogen and subsequently

Abbreviations: Bz-Arg-OET, α-*N*-Benzoyl-L-arginine ethyl ester HCl; Bz-Arg-NOAn, α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide HCl; Bz-Arg-Nap, α-*N*-Benzoyl-DL-arginine-2-naphthylamide HCl.

measuring the trypsin formed [1—3]. One of the disadvantages of this two-stage assay is that it precludes the sensitive determination of enterokinase in biological fluids such as serum, due to the presence of trypsin inhibitors. The failure of these inhibitors to interact with enterokinase (Magee, A.I., unpublished data) should however permit direct measurement of this enzyme. Hesford et al. [4] reported the synthesis of an artificial substrate *tert*-butyloxycarbonyl-tetra-L-aspartyl-L-lysyl-2-naphthylamide; the 2-naphthylamine released was assayed colorimetrically with a diazo dye. This substrate exploits the unusual tetra-aspartyl sequence characteristic of the activation peptides of mammalian trypsinogens [5]. The strongly anionic sequence renders the zymogen a much better substrate for enterokinase than for trypsin; this serves as a safety device against disastrous activation of trypsinogen within the pancreas while favouring the rapid activation of pancreatic juice within the duodenum. Nevertheless since the artificial substrate has a lysine residue on the carbonyl side of the target bond it should, like trypsinogen, be cleaved by trypsin. This paper compares the activities of highly purified enterokinase and trypsin towards glycine-tetra-L-aspartyl-L-lysyl-2-naphthylamide (Gly-$(Asp)_4$-Lys-Nap) and other synthetic trypsin substrates and describes a sensitive assay for enterokinase in serum which avoids interference by nonspecific arylamidases.

Materials and Methods

Gly-$(Asp)_4$-Lys-2-naphthylamide was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland), α-*N*-Benzoyl-DL-arginine-2-naphthylamide HCl (Bz-Arg-Nap), α-*N*-Benzoyl-L-arginine ethyl ester HCl (Bz-Arg-OET), α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide HCl (Bz-Arg-NOAn), twice crystallised bovine trypsin and Diagnostic Kit No. 251 for assaying leucine aminopeptidase were from Sigma Chemical Co. (Poole, U.K.). Trasylol was from Bayer (Haywards Heath, U.K.) and glycodeoxycholic acid was from Calbiochem. (Bishops Stortford, U.K.). Enterokinase was purified as previously described [3].

Optimum pH, Ca^{2+} and glycodeoxycholic acid concentrations for hydrolysis of Gly-$(Asp)_4$-Lys-Nap by enterokinase and trypsin. 1.8 ng pure human enterokinase or 0.5 μg trypsin were incubated for 16 h or 1 h, respectively, at 37°C in 0.2 ml 25 mM Tris-HCl buffer at the appropriate pH and containing 0.5 mM Gly-$(Asp)_4$-Lys-Nap substrate. The amount of 2-naphthylamine released was measured by modification of the method of Goldbarg and Rutenburg [6] using the materials supplied in Sigma Diagnostic Kit No. 251. The reaction was stopped by the addition of 0.1 ml 2 N HCl. 0.1 ml 0.2% sodium nitrite was added followed by 0.2 ml 0.5% ammonium sulfamate 3 min later. After a further 3 min, 0.4 ml 0.05% *N*-(1-naphthyl)ethylenediamine in 95% ethanol was added and the colour developed after 45 min was measured at 580 nm against a distilled water blank. The amount of liberated 2-naphthylamine was estimated from a simultaneous standard curve. In the same way various concentrations of Ca^{2+} (0—20 mM) and glycodeoxycholic acid (1—10 mM) were tested at the optimum pH for their effect on the hydrolysis of Gly-$(Asp)_4$-Lys-Nap by trypsin or enterokinase.

Time course, calibration curves and K_m for Gly-$(Asp)_4$-Lys-Nap hydrolysis. 1.8 ng pure human enterokinase or 0.5 μg trypsin were incubated at 37°C in

0.2 ml 25 mM Tris-HCl (pH 8.4), containing 1.5 mM Gly-$(Asp)_4$-Lys-Nap and either 0.1 mM or 10 mM Ca^{2+}. Hydrolysis was stopped at suitable intervals and the liberated 2-naphthylamine was measured. For the calibration curve aliquots of pure enterokinase at 1—72 ng/ml, or trypsin at 0.5—10 μg/ml, were incubated at 37°C in 0.2 ml 25 mM Tris-HCl (pH 8.4) containing 1.5 mM Gly-$(Asp)_4$-Lys-Nap and either 0.1 mM or 10 mM Ca^{2+}. The K_m for Gly-$(Asp)_4$-Lys-Nap hydrolysis by trypsin and enterokinase was determined between 0.5 and 2.5 mM and the data interpreted by the direct linear plot of Eisenthal and Cornish-Bowden [7]. The trypsin preparation was 50% active by weight as determined by the method of Hixson and Nishikawa [8].

The effect of Trasylol. 0.2 ml aliquots of 1.5 mM substrate solution were made up as described above and containing from 0.1—5 μg Trasylol. The effect on trypsin and enterokinase activity was assessed after appropriate incubation periods.

Hydrolysis of other synthetic substrates. For Bz-Arg-OEt and Bz-Arg-NOAn, 0.1 ml aliquots of substrate (Bz-Arg-NOAn was solubilised in dimethyl sulfoxide), were mixed in a 1 ml cuvette with 0.5 ml 50 mM Tris-HCl (pH 8.4) containing either 0.2 mM or 20 mM $CaCl_2$. The volume was made up to 1 ml with water and the final substrate concentrations were varied between 0.02 and 2.0 mM for Bz-Arg-OEt and 0.5—5.0 mM for Bz-Arg-NOAn. Following the addition of either 1.8 ng enterokinase or 0.5 μg trypsin any hydrolysis at 30°C was followed by changes in absorbance at 253 nm for Bz-Arg-OEt and 405 nm for Bz-Arg-NOAn. For Bz-Arg-Nap, 0.2 ml aliquots of 25 mM Tris-HCl (pH 8.4) containing 10% dimethyl sulfoxide (v/v), either 0.1 mM or 10 mM $CaCl_2$, and substrate at 0.5—2.5 mM, were incubated at 37°C with 0.5 μg trypsin for 1 h or with 1.8 ng enterokinase for 16 h. Liberated 2-naphthylamine was estimated as described above. In all cases kinetic parameters were estimated as described above.

The effect of specific anti-enterokinase antibody on Gly-$(Asp)_4$-Lys-Nap. 20 μl of a 1/50 dilution of a specific rabbit antibody against the protein core of human enterokinase [9] was preincubated at 30°C with 1.8 ng pure enzyme (5 μl) before adding 75 μl water and 100 μl 50 mM Tris-HCl (pH 8.4) containing 1 mM Gly-$(Asp)_4$-Lys-Nap. Control samples omitted either the enzyme or the antiserum. After incubation for 16 h at 37°C, liberated 2-naphthylamine was measured. The same preparation of antiserum was also assessed for its ability to inhibit enterokinase-catalysed activation of trypsinogen as described previously [10].

Inhibition of serum arylamidases. Aliquots of 0.2 M $ZnSO_4$ were added to 0.1 ml of normal mouse serum to give a final molarity in 0.2 ml of 1—5 mM. To this was added 0.1 ml of 3 mM Gly-$(Asp)_4$-Lys-Nap solution in 50 mM Tris-HCl (pH 8.4) containing 0.5 μg Trasylol. Incubation was for 16 h at 37°C; any 2-naphthylamine liberated was measured following brief centrifugation at 2000 $\times g$ to remove serum proteins (which precipitated after addition of the diazo-dye reagent).

Measurement of enterokinase in serum. 5-μl aliquots of pure enterokinase (0.1—7.2 ng enzyme protein) were mixed with 0.09 ml normal mouse serum. 0.1 ml of substrate solution described above was added together with a 5 μl aliquot of 0.2 M $ZnSO_4$. After 16 h at 37°C, the liberated 2-naphthylamine was measured and corrected for a serum control (which contained no enterokinase).

Results

The pH optimum for pure human enterokinase was found to be 8.4 (at 37°C) in 25 mM Tris-HCl buffer. At this pH enzyme activity increased in a hyperbolic fashion with increasing Ca^{2+} concentrations up to 10 mM (Fig. 1). For trypsin the optimum pH was approx. 8.3 but the response to Ca^{2+} was sigmoidal at 0.1—10 mM Ca^{2+} (Fig. 1). At low Ca^{2+} concentrations up to 10 mM glycodeoxycholic acid had no effect on the activity of either enzyme; the effect of this detergent at high Ca^{2+} concentrations could not be assessed as it was precipitated by the cation.

In the presence of either 0.1 mM or 10 mM Ca^{2+} hydrolysis of Gly-$(Asp)_4$-Lys-Nap by enterokinase was linear with time even after 24 h (Fig. 2). The sigmoidal response of trypsin to Ca^{2+} meant that there was a marked Ca^{2+} dependence on the rate of hydrolysis. In the presence of 0.1 mM Ca^{2+} hydrolysis was very slow whereas in 10 mM Ca^{2+} the substrate was completely hydrolysed after 30 min (Fig. 2). Subsequent incubation times were fixed at 16 h for enterokinase at both Ca^{2+} concentrations and 15 min and 16 h for trypsin in 10 mM and 0.1 mM Ca^{2+}, respectively.

The amount of substrate hydrolysed was directly proportional to the quantity of enzyme incubated over a wide range; 1.0—72 ng enterokinase protein/ml for enterokinase and 0.5—10 μg/ml for trypsin (Fig. 3). There was a 200-fold difference in the limits of detection in terms of enzyme protein and this represented approx. 10 000-fold difference in molar amounts. Trasylol had no inhibitory action on enterokinase hydrolysis of Gly-$(Asp)_4$-Lys-Nap up to a concentration of 2.5 μg Trasylol/ml. This represented approx. $5 \cdot 10^3$ molar

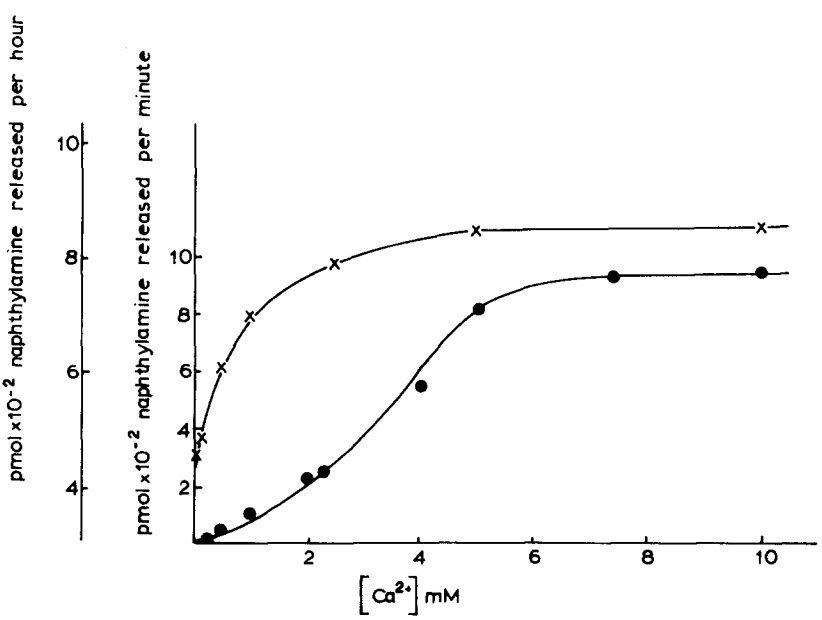

Fig. 1. The effect of Ca^{2+} on enterokinase- and trypsin-catalysed hydrolysis of Gly-$(Asp)_4$-Lys-Nap. X, rate of enterokinase-catalysed hydrolysis in pmol/h; •, rate of trypsin-catalysed hydrolysis in pmol/min.

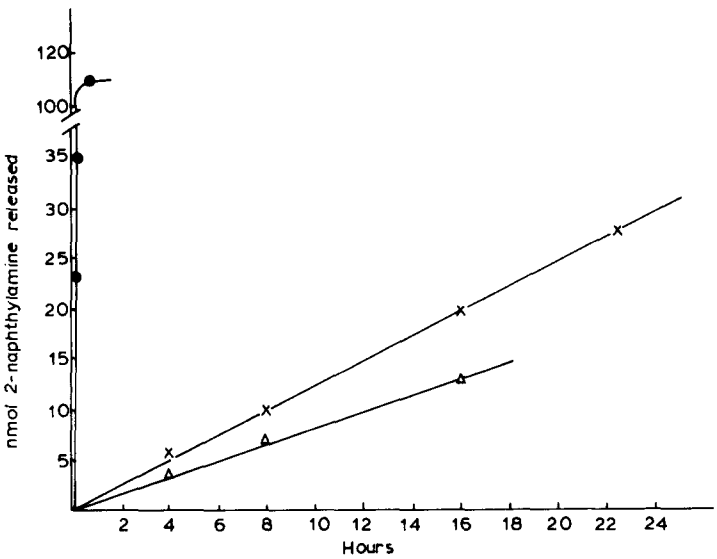

Fig. 2. Time course for the hydrolysis of Gly-(Asp)$_4$-Lys-Nap by trypsin and enterokinase at different Ca^{2+} concentrations. X, enterokinase-catalysed hydrolysis in 10 mM Ca^{2+}; △, trypsin-catalysed hydrolysis in 0.1 mM Ca^{2+}; ●, trypsin-catalysed hydrolysis in 10 mM Ca^{2+}.

excess of Trasylol. At a molar excess of $1 \cdot 10^4$ enterokinase activity was reduced by about 10%. At all Ca^{2+} concentrations tested trypsin activity was almost completely inhibited by Trasylol at a 1 : 2 molar ratio; less than 2% residual activity remained.

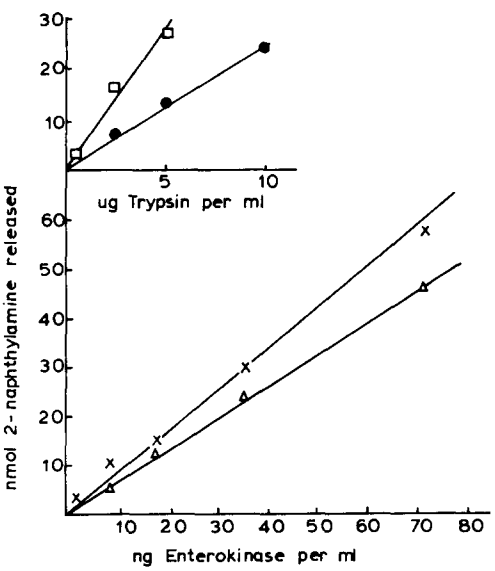

Fig. 3. Calibration curves for enterokinase and trypsin. X, enterokinase in 25 mM Tris-HCl (pH 8.4) containing 10 mM Ca^{2+} and 0.5 μg Trasylol; △, enterokinase in mouse serum (see text for details). Inset. ●, trypsin in 25 mM Tris-HCl (pH 8.3) containing 0.1 mM Ca^{2+}; □, in 10 mM Ca^{2+}.

TABLE I

KINETIC PARAMETERS FOR ENTEROKINASE AND TRYPSIN-CATALYSED HYDROLYSIS OF FOUR SYNTHETIC SUBSTRATES

—, no hydrolysis detected.

Substrate	Ca^{2+}	Enterokinase		Trypsin	
	(mM)	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
Bz-Arg-OEt	0.1	—	—	0.017	21.5
	10.0	—	—	0.017	30.5
Bz-Arg-Nap	0.1	—	—	2.6	0.47
	10.0	—	—	1.65	0.47
Bz-Arg-NOAn	0.1	*	*	3.9	2.72
	10.0	—	—	2.65	2.72
Gly-$(Asp)_4$-Lys-Nap	0.1	0.525	21.5	**	**
	10.0	0.28	28.3	**	**

* $K_m \geqslant 15$ mM.
** Does not demonstrate simple kinetics.

The values of the kinetic parameters K_m and k_{cat} were determined, where possible, for both enzymes against the four substrates described in Materials and Methods (Table I). Bz-Arg-OEt or Bz-Arg-Nap were not hydrolysed by up to 80 ng pure human enterokinase, even after 16 h incubation at 37°C. Bz-Arg-NOAn was only slightly hydrolysed by enterokinase in a low Ca^{2+} environment; this was abolished in 10 mM Ca^{2+}. Although Gly-$(Asp)_4$-Lys-Nap was hydrolysed by trypsin it was not possible to determine any values of K_m; the data indicated that the mechanism of hydrolysis was complex and did not obey Michaelis-Menten kinetics. Specific rabbit anti-human enterokinase antiserum did not abolish hydrolysis of Gly-$(Asp)_4$-Lys-Nap by enterokinase although 25 μl of the same preparation completely inhibited the activation of trypsinogen by 10 ng of enzyme.

Hydrolysis of Gly-$(Asp)_4$-Lys-Nap by nonspecific arylamidases was the main source of interference in the measurement of enterokinase mixed with serum. Inhibition of this activity was complete at concentrations of $Zn^{2+} > 3$ mM. In aqueous solution Gly-$(Asp)_4$-Lys-Nap precipitated at concentrations >2 mM Zn^{2+}, but, when mixed with serum, the addition of Zn^{2+} did not affect this substrate and no inhibition of enterokinase by 5 mM Zn^{2+} was observed. The amount of substrate hydrolysed was directly proportional to the quantity of enzyme incubated, and the limit of detection was again 1 ng of enzyme protein/ml, equivalent to approximately 8 fmol of enterokinase (Fig. 3).

Discussion

The pH optimum for human enterokinase against Gly-$(Asp)_4$-Lys-Nap of 8.4 was 0.5 pH units higher than that described by Hesford et al. [4], for the hydrolysis of *tert*-butyl-oxycarbonyl-$(Asp)_4$-Lys-Nap by the porcine enzyme although both values are close to the pH optimum for activation of trypsinogen by porcine enterokinase [11]. The effect of Ca^{2+} on the activation of trypsi-

nogen by enterokinase has not been fully resolved. Low Ca^{2+} concentrations (1—2 mM) have been shown to enhance enterokinase activity towards its natural substrate [12,13] whereas inhibition by Ca^{2+} concentrations >1 mM have also been reported [11]. Baratti and Maroux [15] have suggested that an extended active site on enterokinase may consist of a cluster of basic amino acids which bind the anionic activation peptide of trypsinogen. Increasing Ca^{2+} concentration, however, might be expected to be inhibitory by neutralising the negative charges on the activation peptide. The availability of a tetra-aspartyl-containing artificial substrate for enterokinase may resolve some of the difficulties in this field. In the present one stage system, hydrolysis of Gly-$(Asp)_4$-Lys-Nap by human enterokinase at pH 8.4 in 10 mM Ca^{2+} was three times faster than in 0.1 mM Ca^{2+}, an increase principally due to a decrease in K_m but also to a rise in k_{cat}. Ca^{2+} would seem therefore to increase the affinity of enterokinase for the tetra-aspartyl-containing substrates as well as promoting dissociation of the product. This agrees with data that Ca^{2+} in some indirect way stabilises a heat labile extended active site on the enterokinase molecule [14].

Enterokinase did not hydrolyse Gly-$(Asp)_4$-Lys-Nap below pH 7. Hydrolysis of trypsinogen by enterokinase, however, is carried out at pH 5.6 since activation of trypsinogen by trypsin becomes increasingly significant at higher pH [2]. Nevertheless the ratio k_{cat}/K_m for enterokinase hydrolysis of trypsinogen at pH 5.6 [2] was of the same order as the k_{cat}/K_m ratio for Gly-$(Asp)_4$-Lys-Nap at pH 8.4. This supports the concept that the tetra-aspartyl sequence of trypsinogen is the high affinity binding site for enterokinase [2].

Trypsin hydrolysis of Gly-$(Asp)_4$-Lys-Nap with increasing concentrations of Ca^{2+} was sigmoidal in response. Under these assay conditions bovine trypsin was found to be exceptionally labile if the Ca^{2+} concentration was 2 mM or less, which agreed with the proposal that active bovine trypsin was stabilised by Ca^{2+} binding to a high affinity site [16,17]. Colomb et al. [18] have shown the same instability for human trypsin at pH 8. Therefore the hydrolytic activity against Gly-$(Asp)_4$-Lys-Nap was superimposed on the autolytic activity and as a result no satisfactory kinetic data was obtained.

Trypsin-catalysed hydrolysis of the other substrates was also Ca^{2+} sensitive. For Bz-Arg-OET the increased rate in 10 mM Ca^{2+} was due to a change in V whereas for Bz-Arg-NOAn and Bz-Arg-Nap the faster hydrolysis was due to a decrease in the respective K_m value. It was not possible to determine satisfactory K_m values for human enterokinase using these artificial trypsin substrates. Other workers have used μg amounts of the porcine enzyme or very high concentrations of substrate in order to demonstrate enterokinase-catalysed hydrolysis of Bz-Arg-OET and Bz-Arg-NOAn; K_m values or 0.12 mM at pH 6.5 and 12.7 mM at pH 8 have been reported for Bz-Arg-OEt and Bz-Arg-NOAn, respectively [14,15]. In order to check that the lack of reaction was not due to denaturation of enterokinase or spontaneous hydrolysis of substrate either trypsin or 0.5 mM Gly-$(Asp)_4$-Lys-Nap was added to control tubes after 16 h and incubation continued for a further 8 h. The appropriate substrate was hydrolysed by trypsin and Gly-$(Asp)_4$-Lys-Nap was hydrolysed as normal by enterokinase.

Bile salts have been reported to enhance the enterokinase activity of intestinal brush border suspensions and solubilised intestinal homogenates [19—21]. In the present study 0.1—10 mM glycodeoxycholic acid had no effect either on

trypsin or enterokinase-catalysed hydrolysis of Gly-$(Asp)_4$-Lys-Nap. The absence of any detergent effect at concentrations above the critical micelle concentration of 4.6 mM [22] is not unexpected for these highly soluble enzymes.

It has been shown previously that rabbit anti-human enterokinase antibody will inhibit the activation of trypsinogen [10] and that the antibody was directed against the protein core of this heavily glycosylated enzyme [9]. In this study the same antibody did not prevent enterokinase-mediated hydrolysis of Gly-$(Asp)_4$-Lys-Nap even though simultaneous controls confirmed total inhibition of trypsinogen-activating ability. This would suggest that the antigenic determinant lies close to, but is not part of, the active site of enterokinase, access to which by the small artificial substrate but not by the larger trypsinogen molecule is preserved in the enzyme-antibody complex.

It is not known whether enterokinase activity may be present in human or mammalian blood but evidence of systemic absorption of intestinal macromolecules with or without mucosal injury suggests that this is a likely event [23,24]. Mouse serum was used in this study as a necessary preliminary to an investigation of the fate of intravenously administered enterokinase; the assay is, however, equally applicable to human serum. The selective inhibition of mouse serum arylamidases [25,26] by 5 mM Zn^{2+} did not interfere with the measurement of enterokinase in serum. Serum trypsin levels are around 300 ng/ml in man [27] and if this is so for the mouse it is well below the limits of detection in this system. α_2-Macroglobulin-bound trypsin can hydrolyse low molecular weight substrates [28] but no endogenous activity was found when Trasylol was omitted from a control tube. The calibration curve for serum enterokinase was in surprisingly good agreement with that obtained in aqueous solution, and after correction for serum blanks only a 10% reduction in corresponding values was seen (Fig. 3).

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