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EFFECT OF IONIC STRENGTH AND CALCIUM IONS ON THE ACTIVATION OF TRYPSINOGEN BY ENTEROKINASE

A MODIFIED TEST FOR THE QUANTITATIVE EVALUATION OF THIS ENZYME

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SUMMARY

The K_m of the activation of trypsinogen by pure enterokinase (enteropeptidase, EC 3.4.4.8) was shown to increase abruptly with ionic strength, with 2 plateaux for $I < 0.15$ and $0.2 < I < 0.3$. This activation is also Ca^{2+}-dependent, probably because a partial conversion of trypsinogen to inert proteins by the formed trypsin is prevented. A modified test for measuring enterokinase activity is proposed.

INTRODUCTION

Enterokinase (enteropeptidase, EC 3.4.4.8), an enzyme of the intestinal brush border membrane[1,2], has recently been shown to be a major factor for the cascade process leading to the *in vivo* activation of trypsinogen and other pancreatic zymogens[3,4]. Any parameters affecting the rate and yield of this most important step of intraluminar digestion deserves special interest.

Kunitz[5] was the first to report that trypsinogen autoactivation during incubation of the zymogen with enterokinase could be avoided by using very dilute solutions and a pH not exceeding 5.6. In the present work, the effect of ionic strength and Ca^{2+} on the enterokinase-catalyzed activation was investigated in detail. Results obtained led us to modify the routine test previously proposed[4] for measuring the activity of this enzyme.

MATERIALS AND METHODS

Enzymes and reagents

Pure porcine enterokinase was prepared from duodenal mucosa according to

Abbreviations: BAEE, *N*-α-benzoyl-L-arginine ethyl ester. TAME, *N*-α-tosyl-L-arginine methyl ester.

a recently described technique[6] including enzyme solubilization by deoxycholate, removal of impurities at acidic pH, chromatography on DEAE-cellulose and gel filtration through Sephadex G-100 and G-200. Bovine trypsinogen (once crystallized, salt free) was purchased from Worthington. All reagents were analytical grade. A stock 70 mM succinate buffer was prepared by adjusting a succinic acid solution to pH 5.6 by 2 M NaOH. In all assays, this buffer was diluted to a final concentration of 28 mM, either by the other reagents or by water.

Enzyme activities

Trypsin was determined at pH 7.8 and 25 °C by the usual potentiometric technique using a recording Radiometer pH-stat and *N*-α-benzoyl-L-arginine ethyl ester (BAEE) or *N*-α-tosyl-L-arginine methyl ester (TAME) (concentration 2 and 25 mM, respectively) as substrate. Under the conditions employed, TAME led to an approximately 10-fold more sensitive determination, because of a more favorable k_{cat} value. Trypsin (BAEE and TAME) units were defined as the amount of enzyme hydrolyzing 1 μmole of substrate per min under the conditions of the assays.

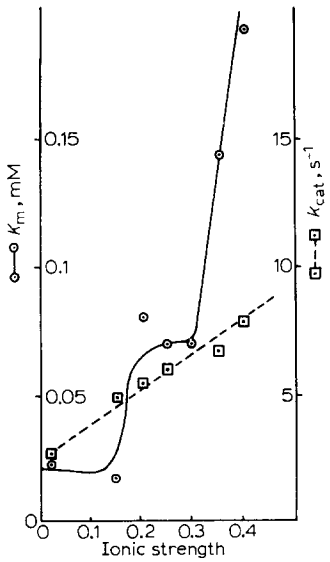
RESULTS

Effect of ionic strength on enterokinase activity

Kinetic parameters k_{cat} and K_m of the enterokinase-catalyzed activation of trypsinogen were derived from 20-min incubation assays in a 20 mM Tris–acetate buffer at pH 5.6. The ionic strength of this buffer was varied by addition of NaCl. In all cases, trypsin activity developed linearly with time during the whole incubation period. The ionic strength dependence of the two parameters is illustrated in Fig. 1. An increase of the ionic strength is seen to induce a sharp K_m increase with 2 plateaux corresponding to K_m values of 0.02 and 0.07 mM, and ionic strength ranges of $I < 0.15$ and $0.20 < I < 0.30$. The catalytic constant k_{cat} is also observed to rise with ionic strength, but more gradually and continuously. Since, for reasons previously given in the Introduction, trypsinogen concentrations much lower than K_m are employed, the net result of the above variations is a marked decrease of the apparent activity of enterokinase at higher ionic strength. These data are in good agreement with the previously reported effect of ions on enterokinase activity[1].

Effect of Ca^{2+}

The effect of Ca^{2+} on trypsinogen activation in a succinate buffer at pH 5.6 is indicated by Fig. 2. Addition of 1 mM Ca^{2+} in the form of $CaCl_2$ is seen to slightly enhance the initial rate (1.4-fold) and the yield of the reaction (1.2-fold) over the blank without added Ca^{2+}. This yield is 100% in the presence of Ca^{2+}, since the maximal specific trypsin activity attained, after 240 min is equal to the potential specific activity (45 μmoles/min per mg) of the Worthington trypsinogen sample used for the assays. By contrast, both initial rate and yield are markedly depressed by addition of 1 mM EDTA. The consequence of these 2 opposite effects is that addition of Ca^{2+} now appears to enhance 3.4-fold the rate and twice the yield of the activation over those observed in absence of the ions. It may be assumed that results of blank assays are affected by traces of Ca^{2+} in the reagents.



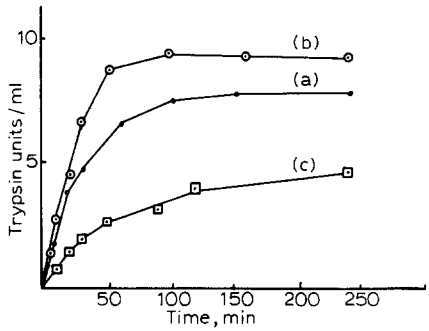


Fig. 1. Ionic strength dependence of trypsinogen activation by enterokinase. Enterokinase was assayed at 25 °C towards bovine trypsinogen solutions in a 20 mM Tris–acetate buffer at pH 5.6 containing varying concentrations of NaCl.

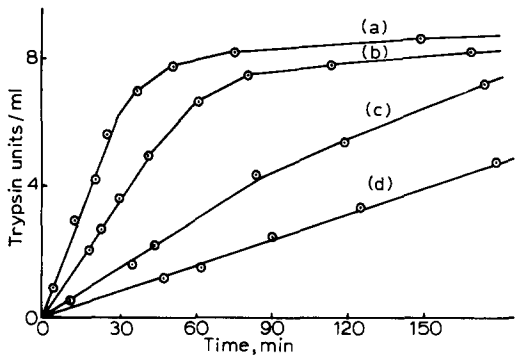
Fig. 2. Rate and yield of trypsinogen activation in the presence or absence of Ca^{2+}. Incubations were performed at 25 °C in a succinate buffer (final concentration, 28 mM) at pH 5.6 containing bovine trypsinogen (0.2 mg/ml) and enterokinase. Curves a, b and c, respectively, succinate alone and with 1 mM added Ca^{2+} or EDTA. Trypsin activity was assayed in samples removed after varying time intervals.

Modified test for enterokinase

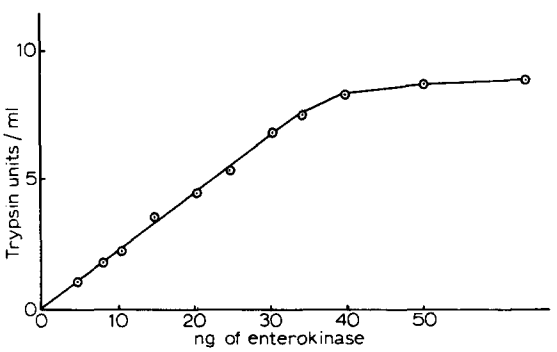
The use of a polyanionic buffer for the quantitative test proposed in our previous publication[4] was dictated by the difficulty to find a monoanionic buffer with a good buffering capacity at pH 5.6. However, the choice made earlier of a 40 mM citrate buffer now appears rather unfortunate, because of the high ionic strength of this buffer at pH 5.6 and its ability to chelate Ca^{2+}. More precisely, the ionic strength of a 40 mM citrate buffer at pH 5.6 is 0.174. This value is situated between the 2 plateaux of Fig. 1, in a region where strong K_m *vs* I variations are noted. As a consequence, the K_m of the reaction is less favorable than it should be and any ionic strength change brought about for instance by addition of the enterokinase sample, may cause serious errors. Moreover, partial chelation of Ca^{2+} by citrate is attested in this case by the fact that initial rates and activation yield in the absence of added Ca^{2+} are much lower in citrate than in succinate. Therefore, the original technique was modified as follows: a 150-mg sample of bovine trypsinogen [potential specific activity about 45 trypsin (BAEE) units per mg] was taken up in 100 ml of precooled 1 mM HCl containing 5 mM $CaCl_2$. A slight turbidity was spun down at 15 000 rev./min for 10 min in the SS-34 rotor of a refrigerated Sorvall centrifuge. The clear supernatant was adjusted to $A_{280\ nm}$ 1.44 (1 mg/ml) by addition of the required volume of HCl–$CaCl_2$ and stored at 4 °C until use.

For the assays, 0.5-ml aliquots of this solution were mixed with 1 ml of a 70 mM succinate buffer at pH 5.6, $(1-x)$ ml of water and x ($x \leqslant 1$ ml) of the entero-

kinase sample under investigation. After a 30-min incubation at 25 °C, the reaction was stopped by addition of 50 μl of 2 M HCl and trypsin activity was measured with the aid of BAEE (or TAME). Representative activity *vs* time curves are reproduced in Fig. 3a for enterokinase concentrations ranging from 3 to 34 ng/ml. In this range, trypsin formation is zero order between 0 and 30 min. The amounts of trypsin formed in 30 min are strictly proportional to enterokinase concentration (Fig. 3b) when activation does not exceed 75% (formation of 7 trypsin units in 30 min). It has been checked that autoactivation of trypsinogen by trypsin concentrations as high as 4 units/ml was undetectable under these conditions. The enterokinase unit (trypsinogen) was defined as earlier[4] to be the amount of enzyme activating under the conditions of the test 1 nmole of trypsinogen in 30 min.



(a)



(b)

Fig. 3. New test for enterokinase determinations. (a) Time course of trypsinogen activation by enterokinase in a succinate buffer at pH 5.6. Curves a, b, c and d: 34, 17, 7 and 3 ng/ml of enterokinase, respectively. For other conditions, see the text. (b) Proportionality between the concentration of enterokinase and trypsin formed in 30 min.

Direct comparison of citrate and succinate tests was carried out by titration of the same enterokinase solution by both methods. The results reported in Fig. 4 show that the new test possesses a 8-fold higher sensitivity because of a lower ionic strength (0.042 instead of 0.174, see Table I) and the presence of Ca^{2+} in the succinate test compared with the citrate test.

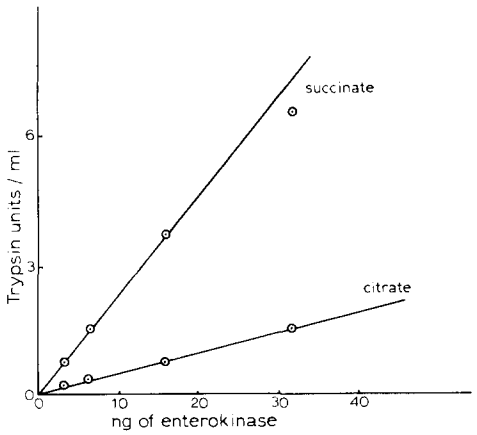


Fig. 4. Comparison between succinate and citrate tests. Proportionality between the concentration of enterokinase and trypsin formed in 30 min. Conditions for the succinate test are given in the text, for the citrate test see ref. 4.

TABLE I

SOME PARAMETERS OF THE BUFFERS USED FOR TRYPSINOGEN ACTIVATION BY ENTEROKINASE

Parameter	*Citrate*[4]	*Succinate (this paper)*	*Maleate*[9]	*Acetate*
Concentration (mM)*	40	28	63	40
Ionic strength*	0.174	0.042	0.040	0.034
pK	5.4	5.6	6.0	4.75
Buffering capacity at pH 5.6**	0.24	0.25	0.25	0.10

* Final values during the test.
** Calculated by $d\alpha/dpH = \alpha\ (1 - \alpha)$ where α is the ionization coefficient.

DISCUSSION

Enterokinase has been recently shown[4] to recognize with a very high degree of specificity the polyaspartyl–lysine structure existing in the N-terminal region of all trypsinogens immediately before the strategic Lys–Ile bond involved in activation. This process may be assumed to result from electrostatic interactions between the charged groups of the above structure and corresponding charged subsites in the enzyme. The fact that electrostatic interactions are affected by ionic strength probably explains the marked ionic strength dependence of the enterokinase-catalyzed activation of trypsinogen. The two plateaux visible on the curve illustrating the K_m variations in Fig. 1 suggest that at least two groups of subsites are present with different sensitivities towards ionic strength.

Ca^{2+} have been known for a number of years to be of great importance for the autoactivation of trypsinogen. More recently, the zymogen has been reported[7] to

possess two binding sites for Ca^{2+}, one with a high affinity preventing the formation of inert proteins through a conformational change in the zymogen molecule, and the other with a lower affinity speeding up the cleavage by trypsin of the strategic Lys–Ile bond. This cleavage in the absence of Ca^{2+} is much slower than normal because of the proximity of the aspartyl residues in the chain of the zymogen[8]. The fact that the hydrolysis of BAEE by enterokinase is not Ca^{2+} (or EDTA) sensitive proves that Ca^{2+} affects trypsinogen rather than enterokinase during the activation of the zymogen by this latter enzyme (unpublished results). The low affinity site is not likely to play here a major role since enterokinase action is normally enhanced by the aspartyl residues[4]. On the contrary, the binding of Ca^{2+} to a high affinity site can be expected to favor the enterokinase-mediated activation by preventing the formation of inert proteins by the generated trypsin. The assumption that this site is predominant in the case of enterokinase is consistent with the low concentration of added Ca^{2+} (1 mM) required to attain the full effect. The role of added Ca^{2+} for trypsinogen activation by enterokinase was not apparent in the first investigation of this series[4] probably because the corresponding site in the zymogen was already saturated by traces of calcium in the reagents.

The above discussed ionic strength and Ca^{2+} effect was taken into account in the new procedure proposed for measuring enterokinase activity. As shown by Table I, the recommended succinate buffer combines a good buffering capacity at pH 5.6 with an ionic strength corresponding to the lower plateau in Fig. 1. Therefore, results may be expected to be sensitive, reproducible and reliable, even when the pH and ionic strength of the investigated enterokinase samples are not optimal. In its present form, the test is approximately 8 times as sensitive as the previous one using citrate, with the consequence that the specific activity of pure enterokinase is now 44 000 units·mg^{-1} (ref. 6) and that enterokinase concentrations lower than 3 ng/ml can be readily measured with BAEE as the substrate for trypsin. This minimal value is further lowered by a factor of 10 when TAME is employed in place of BAEE.

Finally, Table I shows that acetate is not quite satisfactory because of its relatively low buffering capacity at pH 5.6. The recently proposed maleate buffer[1,9] can be expected, when used at a slightly higher concentration, to lead to similar results as those attained in the presence of succinate.

The origin and extent of the positive effect of bile salts[10] on trypsinogen activation by enterokinase has not been investigated.

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