THE RENNIN CATALYZED HYDROLYSIS OF A TRIPEPTIDE
Evidence for Catalysis by Two Carboxyl Groups

Gerald E. Clement and G. S. Cashell
Department of Chemistry, Kenyon College
Gambier, Ohio 43022

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SUMMARY: The kinetics of the rennin catalyzed hydrolysis of Z-His-Phe-Trp-OEt* to produce Z-His-Phe and Trp-OEt in the acid pH range have been measured. The pH-$E_o k_{cat}/K_m$ curve is bell shaped with pKa's 2.9 and 5.4. The decrease in the k_{cat}/K_m values at pH's < 4 seems to be due to large increases in K_m with decreasing pH. Rennin was found to be unstable below pH 2 with good stability exhibited between pH 2 and 6. A method for chromatographing rennin and determining its milk clotting activity is presented.

Rennin, the predominant milk clotting enzyme found in the fourth stomach of the calf, is distinguished by its large milk clotting activity in proportion to its proteolytic activity[1]. Rennin acts in limited proteolysis to cleave only the Phe-Met bond in K-casein thereby causing the milk to clot. Hill[2] has synthesized several peptides containing the sensitive Phe-Met bond and found that only the pentapeptide Ser-Leu-Phe-Met-Ala-OMe was split by rennin. The rate was still three orders of magnitude less than that of K-casein. This rate of splitting of K-casein appears to be a bell shaped function of pH with the maximum between pH 5 and 6.

In this study we have investigated the pH dependency of the rennin catalyzed hydrolysis of Z-His-Phe-Trp-OEt, a substrate carrying a positively charged group throughout this pH range. No clear pH dependencies have been reported for rennin catalyzed hydrolyses. The results reported herein make possible a direct comparison between rennin and pepsin[3,4].

EXPERIMENTAL: Rennin (Lot #11) was purchased from Miles Laboratory. Z-His-Phe-Trp-OEt lot G 1030 and Z-His-Phe lot G 1270 were Cyclo Chemical while Trp-

* Z-His-Phe-Trp-OEt is N-benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophanethyl ester.

OEt was obtained from Mann. All buffers were prepared from reagent grade materials and double distilled water. The milk was Carnation, instant non-fat dry milk.

The kinetic studies were carried out as previously described for pepsin[5]. The point of cleavage of the Phe-Trp bond was determined by TLC on the reaction mixture (solvent 95-chloroform, 5-methanol, 1-acetic acid) and by kinetically doing infinity runs (100 ± 2%). Rennin and prorennin* were purified using much the same method as for pepsin[6]. Approximately 100 mg. of commercial rennin or prorennin was dissolved in 6 ml. of 0.1 N pH 5.2 acetate buffer at 25° which was then lowered to pH 2 by adding hydrochloric acid. After 20 min. the pH was increased to 5.2 by the addition of concentrated sodium acetate solution. The resulting rennin solution was chromatographed on a 2x 30 cm SE-Sephadex C-25 column with a 0.4 M pH 5.2 acetate buffer. Four ml. fractions were collected with all active rennin found in fraction numbers 10-15. For commercial rennin 2 large non-active peaks were observed in fractions 25-37 plus a small peak in the NH_3 wash[6]. From the prorennin samples this large non-active peak was not observed. The amino acid analysis of the rennin fractions agreed with previous results within ± 1-2 residues[1]. The milk clotting activity of the rennin samples from the chromatography as measured by (1/clotting time per ml rennin solution) was determined using the method of Bundy[7] in a weakly buffered milk solution.

Table I shows initial milk clotting time as a function of the pH of the rennin milk clotting solution. It is obvious that the clotting time varies

Table I: Clotting Time of Rennin as a Function of pH at 37°

Final pH of Milk Solution after Addition of Rennin	Clotting Time (sec)
5.10	60
5.30	92
5.44	111
5.51	122
5.62	160
5.75	195

* The preparation of prorennin from fresh calf stomachs will be reported in a subsequent publication.

widely as a function of pH. Table II shows that rennin is reasonably stable in the pH range 2-6 and quite unstable below pH 2 especially in the presence of chloride ion. Rennin is completely stable for at least one month as a frozen sample in the chromatography buffer. To obtain this data a constant amount of rennin was dissolved in the buffers at the various pH's and the milk clotting activity determined on 1 ml. aliquots. For these measurements the buffering capacity of the milk solution was increased 4 times

Table II: Stability of Rennin at 37°

pH Buffer	% Rennin Activity Remaining after 9 Hours
1.2 HCl	15
1.2 H_2SO_4	25
2.1 citrate (0.1M)	80
3.0 citrate (0.1M)	93
4.0 citrate (0.1M)	83
4.2 acetate (0.1M)	91
4.7 citrate (0.1M)	84
5.2 acetate (0.4M)	97
5.3 citrate (0.1M)	84
5.8 citrate (0.1M)	87
6.0 phosphate (0.1M)	95

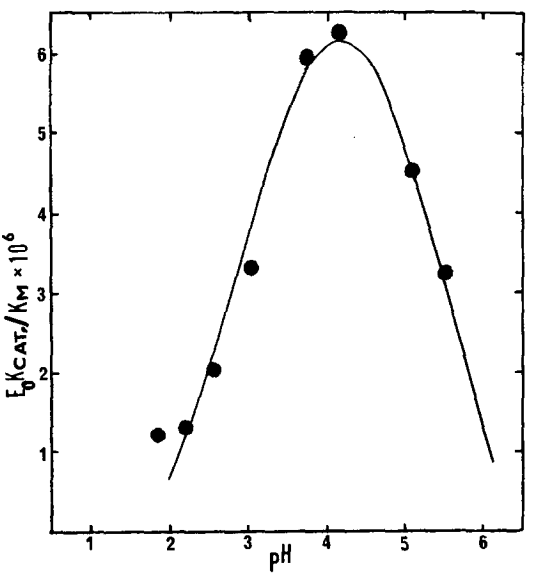

Fig. 1. pH-dependence of $E_o k_{cat}/K_m$ for the Pepsin Catalyzed Hydrolysis of Z-His-Phe-Trp-OEt at 37°
(So) = 1.52×10^{-4}M; (Eo) = 1.9 in $(\text{clotting time})^{-1}(\text{ml})^{-1}$

RESULTS AND DISCUSSION: Rennin and pepsin both being gastric proteases exhibit many similarities such as maximum activity in the pH region 1-6[1], inhibition by diazo compounds[8], approximately 70% homology in amino acid sequences[9,10], similar molecular weights (30,700 vs. 34,100) and type of activation from the respective zymogen. Therefore, it is significant that the results of the kinetic study on the rennin catalyzed hydrolysis of Z-His-Phe-Trp-OEt shown in Fig. I and Table III are similar to those obtained with pepsin[3,4].

Z-His-Phe-Trp-OEt is a substrate for rennin exhibiting an identical point of cleavage as pepsin. The estimated k_{cat} for the rennin hydrolysis at pH 4.4 is ca. 25 times less than that for pepsin[3]. The k_{cat}/K_m-pH profile is bell shaped (pK's 2.9, 5.4) in agreement with what has been observed for

Table III: Kinetic Constants for Rennin Catalyzed Hydrolyses

pH	$k_{cat}E_o \times 10^8$	$K_m \times 10^3$ M
4.40	2.4	3.2
4.10	2.0	3.6
3.60	1.5	3.9
2.50	1.2	9.5
1.90	1.1	10.0

E_o in units 1/C.T/Ml = 1.0; k_{cat} in sec^{-1}; $S_o = 1.5\text{-}0.2 \times 10^{-3}$M
All buffers were 0.1 M citrate.
The lack of substrate solubility precluded additional measurements above pH 4.4.

pepsin catalysis on similar cationic substrates[3]. This means that there are two important ionizable groups (presumably carboxyl) on the free enzyme. The k_{cat} decreases with pH while the K_m shows large increases. This means that the left hand side of the pH-$k_{cat}E_o/K_m$ curve is largely due to the rapidly increasing K_m. In contrast, the K_m for pepsin is constant in this pH range. This difference in the pH dependency of k_{cat}/K_m and k_{cat} for rennin could be an indication of a multistep reaction. A multistep reaction has long been postulated for pepsin[11] and the identical behavior of these two enzymes suggest a very similar mechanism.

Finally, Ac-Phe-Tyr-OMe exhibits rennin acitivity over an 8 hour period

while the two similar neutral dipeptides Ac-Tyr-Phe-OMe and Ac-Phe-Phe-OMe are completely stable. Further studies with these neutral substrates must await a more active rennin preparation.

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