Studies on Tissue Protease

II. Influence of Metal Salts upon Tryptase Activity of Rabbit Liver

(Directed by Asst. Prof. M. Hayakawa)

By

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Activation by cysteine of catheptase activity is well known, and its activating mechanism has been discussed by many scholars without resulting in definite theory. In this connection influences of various metal salts upon catheptase activity has also been made. Krebs¹⁾ reported that gelatin hydrolysis through catheptase of swine kidney extract has been inhibited by Cu, Ag, Au, Zn, Cd, or by Hg. According to Nakayama²⁾ who studied catheptic kidney proteolysis, CuSO₄ or HgCl₂ was most strongly inhibitory. This was also the case when catheptic proteolysis of swine lung was studied by Onishi³⁾, by whom it was shown that cysteine subdued the inhibitory action of Ag, Cu, or Hg but that no activation by FeSO₄ or FeCl₃ had occurred.

On the other hand Hayakawa⁴⁾ and Ito⁵⁾ found that tryptase of human gastric mucosa and rabbit liver, contrary to catheptase, underwent no activation or seemed to be rather inhibited by cysteine. So influence of heavy metal salts upon tryptase is expected to differ from those upon catheptase.

EXPERIMENTAL

Method

The methods are the same as those previously described⁴⁾.

The enzyme solution was prepared from liver, from which blood had been removed by means of washing through portal vein with aqua dest.

Reaction mixture: 20 cc. of substrate-buffer (pH 7.8) solution and 3 cc. of enzyme solution were mixed with 3 cc. of solution of various metal salts and then incubated under toluene at 37°C for digestion. Molar concentrations of salt in the digestion mixture are given in the Tables.

For controlling 3 cc. of metal salt solution is substituted with buffer

solution of equal quantity. On the other hand, general control experiments without substrate were carried out with and without metal salt under same condition. Increased activity with respect to each 4 cc. of digestion mixture is formol-titrated with $N/10$ -NaOH, and corrected values with general control values are tabulated.

Results with Discussion

$ZnSO_4$ or $Fe_2(SO_4)_3$ appears, in concentration of $1/4,000 M$, already somewhat to retard the hydrolysis amount after 120 hours' incubation of casein or gelatin, and $HgCl_2$ or $CuSO_4$ appears so in that of $1/2,000 M$. (Table I). Inhibitory effects of these metal salts are first manifestly observed in concentration of over $1/500 M$. These inhibitory effects appear to become the more obvious when the times of incubation is the longer.

Inhibitory effect of $AgNO_3$, $NiSO_4$ or of $CoCl_2$ seems less marked

TABLE I
Influence of $ZnSO_4$, $Fe_2(SO_4)_3$, $HgCl_2$ or $CuSO_4$ upon Liver
Tryptase Activity of Rabbit at pH 7.8

Increases of acidity in 4 cc. digestion mixture: cc. of $0.1 N$ NaOH.

Salt	Substrate	Time in hrs.	Molar concentration of salts						
			0	$1/8,000$	$1/4,000$	$1/2,000$	$1/1,000$	$1/500$	$1/1000$
$ZnSO_4$	Casein	24	0.15	0.15	0.1	0.1	0.1	0.05	0.05
		72	0.25	0.25	0.2	0.2	0.15	0.1	0.1
		120	0.4	0.35	0.25	0.25	0.2	0.15	0.15
	Gelatin	24	0.15	0.1	0.1	0.1	0.1	0.05	0.05
		72	0.25	0.15	0.15	0.15	0.15	0.1	0.1
		120	0.3	0.2	0.2	0.15	0.15	0.15	0.15
$Fe_2(SO_4)_3$	Casein	24	0.1	0.1	0.05	0.05	0.05	0.05	0.05
		72	0.25	0.25	0.25	0.2	0.15	0.1	0.1
		120	0.45	0.45	0.35	0.35	0.3	0.25	0.25
	Gelatin	24	0.1	0.05	0.05	0.05	0.05	0.05	0.05
		72	0.2	0.15	0.15	0.15	0.1	0.1	0.1
		120	0.3	0.25	0.2	0.2	0.2	0.15	0.15
$HgCl_2$	Casein	24	0.1	0.1	0.1	0.1	0.05	0.05	0.05
		72	0.25	0.25	0.25	0.25	0.1	0.1	0.1
		120	0.3	0.3	0.25	0.2	0.15	0.15	0.15
	Gelatin	24	0.15	0.15	0.1	0.05	0.05	0.05	0.05
		72	0.15	0.15	0.1	0.1	0.05	0.05	0.05
		120	0.2	0.2	0.2	0.2	0.1	0.1	0.1
$CuSO_4$	Casein	24	0.1	0.1	0.1	0.05	0.05	0.05	0.05
		72	0.2	0.2	0.15	0.1	0.1	0.1	0.1
		120	0.3	0.3	0.3	0.2	0.1	0.1	0.1
	Gelatin	24	0.05	0.05	0.05	0.05	0	0	0
		72	0.2	0.2	0.2	0.1	0.1	0.05	0.05
		120	0.25	0.25	0.25	0.15	0.15	0.1	0.1

than above, because retardation of hydrolysis is observed only by 120 hrs.' digestion or in concentration of over 1/500 *M*. (Table II).

FeSO₄, K₃Fe(CN)₆ and CdSO₄ neither inhibit nor activate (Table III).

TABLE II

Influence of AgNO₃, NiSO₄ or CoCl₂ upon Tryptase
Activity of Rabbit Liver at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Salt	Substrate	Time in hrs.	Molar concentration of salts						
			0	1/8,000	1/4,000	1/2,000	1/1,000	1/500	1/100
AgNO ₃	Casein	24	0.1	0.1	0.1	0.1	0.1	0.1	0.1
		72	0.25	0.25	0.2	0.15	0.15	0.15	0.1
		120	0.3	0.25	0.25	0.15	0.15	0.15	0.15
	Gelatin	24	0.05	0.1	0.1	0.05	0.05	0.05	0.05
		72	0.15	0.1	0.1	0.1	0.1	0.1	0.05
		120	0.25	0.25	0.2	0.2	0.2	0.2	0.15
NiSO ₄	Casein	24	0.1	0.1	—	0.1	—	0.05	0.05
		72	0.2	0.2	—	0.15	—	0.05	0.05
		120	0.3	0.3	—	0.25	—	0.2	0.1
	Gelatin	24	0.05	0.05	—	0.05	—	0.05	0.05
		72	0.15	0.1	—	0.1	—	0.05	0.05
		120	0.2	0.1	—	0.1	—	0.05	0.05
CoCl ₂	Casein	24	0.1	0.1	—	0.1	—	0.1	0.05
		72	0.2	0.2	—	0.15	—	0.1	0.1
		120	0.3	0.3	—	0.2	—	0.15	0.15
	Gelatin	24	0.05	0.1	—	0.05	—	0.05	0.05
		72	0.15	0.15	—	0.1	—	0.05	0.05
		120	0.25	0.3	—	0.15	—	0.05	0.05

It is, however, noteworthy that MnSO₄ seems, in concentration of over 1/2,000 *M*, by 72–120 hours' digestion, to be accelerating for casein or gelatin hydrolysis (Table IV). The effect of K₄Fe(CN)₆ is, in concentration of over 1/2,000–1,000 *M*, similarly accelerating (Table IV). But there is some apprehension that one might confuse enzymes, for which activity manganese sulfate is in fact activating, because enzyme solution used contains not only tryptase but also catheptase or ereptase, and manganese sulfate is known to activate dipeptidase activity. Avoiding such confusion an activating effect of manganese sulfate for tryptase is, however, evidenced because the same results are obtained from another experiment (Table V), for which enzyme solution, made by means of acid treatment⁵⁾, exclusive of catheptase, ereptase and dipeptidase (Table V–b) is employed.

Next manganese sulfate was examined for its effect in the presence of

TABLE III
Influence of FeSO_4 , $\text{K}_3\text{Fe}(\text{CN})_6$ or CdSO_4 upon Tryptase
Activity of Rabbit Liver at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Salt	Substrate	Time in hrs.	Molar concentration of salts				
			0	1/5,000	1/1,000	1/500	1/100
FeSO_4	Casein	24	0.05	0.1	0.1	0.1	0.1
		72	0.15	0.15	0.2	0.2	0.2
		120	0.2	0.2	0.25	0.25	0.2
	Gelatin	24	0.05	0.05	0.05	0.05	0.05
		72	0.1	0.05	0.05	0.05	0.05
		120	0.15	0.1	0.1	0.1	0.2
$\text{K}_3\text{Fe}(\text{CN})_6$	Casein	24	0.15	0.15	0.15	0.15	0.15
		72	0.25	0.25	0.25	0.25	0.25
		120	0.35	0.35	0.35	0.35	0.35
	Gelatin	24	0.05	0.05	0.05	0.1	0.1
		72	0.15	0.15	0.15	0.15	0.15
		120	0.2	0.15	0.15	0.15	0.15
CdSO_4	Casein	24	0.1	0.1	0.1	0.1	0.05
		72	0.15	0.15	0.15	0.1	0.15
		120	0.25	0.2	0.2	0.15	0.2
	Gelatin	24	0.1	0.05	0.05	0.05	0.05
		72	0.15	0.1	0.05	0.05	0.05
		120	0.25	0.2	0.2	0.2	0.2

dialyzed albumin solution, and activating effect observed was duplicated, i.e. the hydrolysis amount became larger than when each of these two activators alone was present (Table VI). But manganese sulfate appears to be somewhat inferior to dialyzed albumin solution in activating effect. From this the suggestion can be made that manganese sulfate may have its own mechanism for activation other than that of dialyzed albumin solution.

Mode of influences of heavy metal salts upon tryptase activity seems, in comparison with those upon catheptase, characteristic in the following respects: Their influences upon tryptase, either activating or inhibiting, appear to be rather delayed, because their influences become more obvious as the time of digestion is increased. Retardations of catheptic hydrolysis are, during the lapse of the time of digestion, more or less restored²⁻³⁾. And the metal salt which is strongly inhibitory against catheptic hydrolysis appears to be not always so against tryptic hydrolysis. Therefore these dissimilarities together with the effects of cysteine, suggest that the mechanism of each of these enzyme actions may differ.

On the other hand tryptase in rabbit liver can be definitely said to exist and it manifests itself highly after duplicate activation through dialyzed

albumin solution and manganese sulfate together.

SUMMARY

Influences of metal salts upon liver tryptase activity were investigated with following results:

1. ZnSO_4 , Fe_2SO_4 , HgCl_2 or CuSO_4 is inhibitory to casein or gelatin hydrolysis through rabbit liver tryptase by 72–120 hours' digestion in concentration of over $1/2000\ M$.

2. The inhibitory effect of AgNO_3 , NiSO_4 or CoCl_2 is less than that of the above, and FeSO_4 , $\text{K}_3\text{Fe}(\text{CN})_6$ and CdSO_4 neither inhibit nor activate.

TABLE IV

Influence of MnSO_4 or $\text{K}_4\text{Fe}(\text{CN})_6$ upon Tryptase Activity of Rabbit at pH 7.8

Increases of acidity in 4 cc. digestion mixture are expressed in cc. of 0.1 N NaOH

Salt	Substrate	Time in hrs.	Molar concentration of salts						
			0	1/8,000	1/4,000	1/2,000	1/1,000	1/500	1/100
MnSO_4	Casein	24	0.1	0.1	0.1	0.1	0.15	0.15	0.15
		72	0.2	0.25	0.25	0.3	0.3	0.35	0.35
		120	0.3	0.3	0.35	0.4	0.4	0.45	0.45
	Gelatin	24	0.1	0.15	0.1	0.1	0.1	0.1	0.1
		72	0.15	0.15	0.15	0.2	0.2	0.25	0.25
		120	0.15	0.2	0.2	0.25	0.25	0.3	0.3
$\text{K}_4\text{Fe}(\text{CN})_6$	Casein	24	0.2	0.2	0.2	0.15	0.15	0.2	0.15
		72	0.25	0.25	0.3	0.35	0.35	0.35	0.35
		120	0.3	0.3	0.3	0.4	0.35	0.4	0.45
	Gelatin	24	0.1	0.1	0.1	0.1	0.1	0.1	0.1
		72	0.2	0.2	0.25	0.25	0.2	0.25	0.25
		120	0.2	0.25	0.25	0.25	0.3	0.3	0.3

TABLE V

a. Influence of MnSO_4 upon Enzyme Solution free from Ereptase and Catheptase Activities

Increased acidity in 4 cc. digestion mixture; cc. of 0.1 N NaOH.

5 cc. enzyme solution was used. pH of digestion, 7.8 Substrate, casein

Time in hrs.	Molar concentration of MnSO_4				
	0	1/10,000	1/5,000	1/1,000	1/100
24	0.15	0.15	0.15	0.2	0.2
72	0.25	0.3	0.3	0.4	0.4
120	0.35	0.35	0.45	0.5	0.6

b. Proteolytic Activity at pH 7.8 of Tissue Homogenate of Rabbit Liver, exposed to pH 3.5 at 37°C for 40 minutes

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 N NaOH.

Substrate	Time in hrs.	pH of digestion	
		7.8	4.5
Casein	24	0.15	0
	72	0.25	0.05
	120	0.35	0.05
Gelatin	24	0.1	0
	72	0.2	0
	120	0.25	0
Pepton	24	0	0
	72	0	0
	120	0	0
M/20-Diglycin	24	0	0
	72	0	0
	120	0	—

TABLE VI

Activation of Rabbit Liver Tryptase with combined Use of dialyzed Albumin solution and Maganese sulfate solution

10 cc. 2%-casein-buffer solution + 10 cc. dialyzed ov/serum albumin solution or buffer solution + 3 cc. enzyme solution + 3 cc. MnSO_4 or buffer solution. Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 N NaOH, pH of digestion 7.8.

Time in hrs.	without albumin and MnSO_4	with dialyzed serum albumin	with dialyzed ovalbumin	with M/100- MnSO_4	with dialyzed serum albumin and MnSO_4	with dialyzed ovalbumin and MnSO_4
24	0.15; 0.1	0.4	0.45	0.35	0.6	0.6 ; 0.5
72	0.25; 0.3	0.65	0.75	0.5	1.05	0.75; 0.75
120	0.35; 0.4	0.9	0.9	0.7	1.35	1.5 ; 1.4

3. MnSO_4 or $\text{K}_4\text{Fe}(\text{CN})_6$ activates casein hydrolysis by 72 hours' digestion in concentration of over 1/1000 M. Such effect is recognizable even when catheptase and ereptase are removed by means of acid treatment⁶⁾, and manganese sulfate is still activating for tryptase activity which is activated simultaneously in the presence of dialyzed albumin solution. In such a way tryptase is, in its highly active state, undoubtedly recognizable on rabbit liver.

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