A Serum Peptidase and Its Possible Role in Protease Inhibition. (22222)

GEORGES UNGAR AND BARRY ADLER.

Department of Pharmacology, U. S. Vitamin Corporation, Yonkers, N. Y.

The presence of a number of peptidases in serum has been detected by the use of synthetic substrates such as leucyl-glycyl-glycine, glycyl-glycyl-glycine, glycyl-leucine, etc. The function of serum peptidases, however, is still largely unknown. The method described by Gomori(1,2), using a chromogenic substrate, is sensitive and simple enough to lend itself easily to physiological and clinical studies. The present paper reports the results of investigations carried out with Gomori's method. These results point to the possibility that part of the well-known antiproteolytic activity of serum is due to enzymatic destruction of proteases.

Methods. Determination of enzyme activity is based on the color reaction between beta-naphthylamine and certain diazotized dyes. When glycyl-beta-naphthylamine is hydrolyzed, the amine set free gives a purple colored compound in combination with the dye. Intensity of color, measured spectrophotometrically, indicates degree of hydrolysis of substrate. *Reagents.* 1. Glycyl-beta-naphthylamine (GBNA) was prepared according to Gomori(2) and used at concentration of 0.0002 M (sample used was prepared and kindly supplied by A. J. Merritt, U. S. Vitamin Corp.). 2. Veronal buffer, pH 7.4, 0.15 M in physiological saline. 3. Acetate buffer, pH 4.2, 1 M, containing 10% Tween 20. 4. Red B Salt (Matheson, Coleman and Bell) in 0.25% solution, to be prepared shortly before use. *Procedure.* The enzyme solution is made up to 6 ml with veronal buffer and placed in water bath at 37.5°C. The reaction is started by addition of 2 ml of GBNA solution. After required interval, usually 30 min., the reaction is stopped by adding 1 ml of Tween-acetate buffer solution. The purpose of Tween 20 is to solubilize the colored compound formed when 1 ml of dye is added to the reaction mixture. Color develops rapidly and remains stable for over an hour. Test solutions are read in Coleman Universal spectrophotometer at 550 mμ against blank containing all reagents except the enzyme. *Color reaction* has a high sensitivity and may be made even more sensitive by reducing volume of reagents. The beta-naphthylamine-dye compound has a molar extinction coefficient of 43800 at 550 mμ. With this procedure 2.5% hydrolysis can still be measured (0.01 μM in final volume of 10 ml). Results of experiments are expressed in terms of μM of substrate hydrolyzed. *Inhibition of protease* by serum was measured by means of method described previously(3). Blood samples were collected by venous or heart puncture and allowed to clot at 37.5°C. Unless otherwise stated, the serum was used shortly after separation. When kept for longer periods, serum was frozen to –20°C. Tissue extracts were prepared according to method described by Fruton(4) and extracts were kept frozen.

Results. Serum samples from laboratory animals and healthy human subjects were tested for peptidase activity on GBNA. Table I shows mean values of GBNA hydrolysis by sera from different species at stated concentrations. Although enzyme activity varied from one species to another it was remarkably constant within the same species.

Kinetic studies were carried out with guinea pig serum because of its high activity. They showed that rate of GBNA hydrolysis decreased as the reaction proceeded (Fig. 1).

TABLE I. Hydrolysis of GBNA by Sera from Different Species.

Species	Conc., ml	μM hydrolyzed in 30 min.	± S.E.	No.
Guinea pig	.1	.089	.004	24
Human	.3	.024	.005	4
Mouse	.3	.020	.005	3*
Rabbit	.5	.062	.008	6
Dog	.5	.032	.005	4
Rat	.5	.028	.002	12

* 3 pooled samples from 4 mice each.

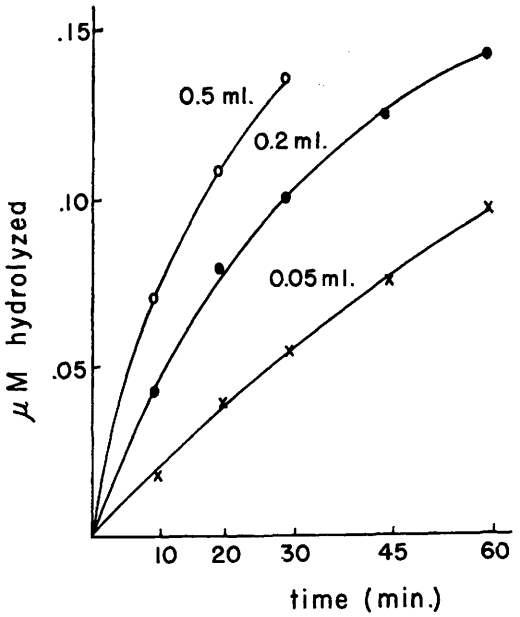

FIG. 1. Hydrolysis of GBNA by guinea pig serum. Abscissa: duration of the reaction (min.); ordinate: μM of substrate hydrolyzed by the amounts of serum indicated.

It was found also, as shown in Fig. 2, that hydrolytic activity was not linearly related to enzyme concentration but approximated more closely log [E]. Rate of hydrolysis, however, was proportional to substrate concentration within limits of experiment. The reaction is not inhibited by its end-products: neither glycine nor beta-naphthylamine have any inhibitory action on the enzyme at the concentrations at which they are released during the reaction.

Hydrolysis of GBNA by guinea-pig serum was inhibited by cyanide and ethylenediamine-tetra-acetic acid (EDTA). This suggested that the enzyme action requires some metallic ion. None of the ions tried (Ca, Mg, Mn, Co, Ni, Cu, Zn), however, activated hydrolysis or reversed action of the chelating agent. Most of the metals tried inhibited GBNA hydrolysis, some of them (Cu, Zn) at very low concentrations.

Enzyme action was also inhibited by a number of amines (methylamine, tryptamine, epinephrine, norepinephrine, histamine, 5-hydroxy-tryptamine). The amino-acids corresponding to some of them (histidine, tryptophan) were inactive. Ammonium salts can also inhibit peptidase; quaternary ammonium compounds (choline, acetylcholine), however, had no inhibitory action. The basic protein, protamine, had a comparatively strong inhibitory action; on molar basis approximately 10^{-7} would be required for 50% inhibition.

The GBNA-hydrolyzing enzyme was found in the albumin fraction when serum was diluted 20-fold with distilled water and precipitated at pH 5.4. Heating serum to 56°C for 30 minutes did not significantly decrease the enzyme activity. Dialysis in the cold for 48 hours against saline did not cause loss of activity. Serum which was kept frozen for 24 hours showed consistently higher activity (by 15 to 25%) than fresh serum.

A few experiments with tissue extracts showed that guinea-pig lung and skin were able to hydrolyze GBNA (0.130 μM by 100 mg of skin) while rat tissues were inactive. Human urine showed slight activity (0.016 μM/ml). It appeared that the GBNA-hydrolyzing enzyme had striking similarities with the substances variously designated as antitrypsin, antifibrinolysin, antiplasmin, protease inhibitor, etc. GBNA hydrolysis and plasmin (fibrinolysin) inhibition follow similar reaction kinetics: the rate is highest in the

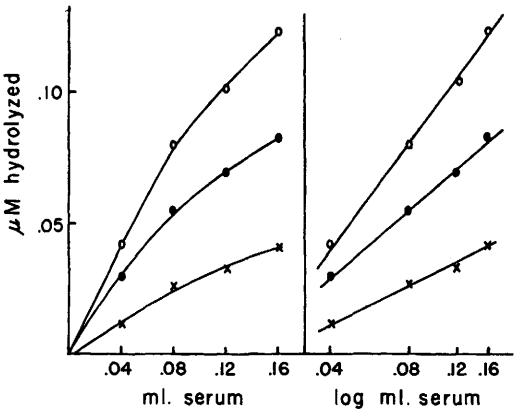

FIG. 2. Hydrolysis of GBNA as a function of enzyme concentration. Ordinate: μM of substrate hydrolyzed in 30 min. at the substrate concentration indicated; abscissa: amount of guinea pig serum added to the substrate (ml) at arithmetic (left) and logarithmic (right) scale.

× = .2 μM GBNA
● = .4 " "
○ = .6 " "

TABLE II. Inhibition of GBNA Hydrolysis by Fibrinolysin and Trypsin.

Inhibitor,* mg		μM hydrolyzed in 30 min. by .1 ml guinea pig serum	.2 ml guinea pig serum
	0	.085	.110
Plasmin	10	.078	.096
	25	.060	.076
	50	.040	.045
Trypsin	0.4	.082	.104
	1.0	.061	.080
	2.0	.037	.046

* Conc. for total volume of 8 ml.
Serum and inhibitor were left in contact for 10 min. before addition of substrate.

first 5 min. and increases linearly with the log of serum concentration(3,5). Both reactions are inhibited by EDTA and inhibition is not reversed by addition of Ca(6). Both systems are inhibited by low concentrations of Cu and protamine(7), by certain amines and ammonia(8). On isoelectric precipitation, both properties of serum are found in the supernatant(9).

One more evidence is supplied by the observation that trypsin (Tryptar, Armour Laboratories) and plasmin (bovine preparation kindly supplied by Dr. E. C. Loomis, Parke, Davis and Co.) inhibit hydrolysis of GBNA by guinea pig serum (Table II). Several other globulin fractions from guinea pig, rabbit and human serum showed no inhibitory action. Plasminogen did not inhibit but inhibitory activity appeared after activation with streptokinase. Kinetic studies of this inhibition are now in progress; they indicate that it is competitive and reversible. Reversibility of the inhibition was substantiated by the following experiment. Guinea pig serum was incubated with plasmin for 30 min. The mixture was then diluted with distilled water and the globulin fraction precipitated out at pH 5.4. Plasmin was in the precipitate while GBNA-peptidase remained in solution. By testing the 2 fractions, it was seen that plasmin was partly destroyed but the peptidase was completely recovered. GBNA and proteases seem to be competing substrates for the same enzyme.

The GBNA-hydrolyzing enzyme, however, is not the only protease-inhibiting factor in serum. There are probably two(10) and possibly three(8) antiproteases in human serum. Table III shows the correlation between plasmin inhibition and GBNA hydrolysis by sera from different species. It is seen that in guinea pigs and possibly rabbits the plasmin-inhibiting action of serum may be due to GBNA-peptidase. In other species, particularly in man, other inhibitors may play a more important role. Bovine serum has no action on GBNA and purified bovine antiplasmin prepared by Loomis(11) also fails to act on this substrate.

The plasmin inhibitor of guinea-pig serum controlled by pituitary and adreno-cortical hormones(3,12) may be identical with GBNA-peptidase. Table III shows that sera from guinea pigs treated with cortisone (5 mg/kg, 4 hours before bleeding) had an increased action on GBNA hydrolysis and plasmin inhibition. The opposite was observed in sera of animals treated for 4 days with 5 mg/kg of pituitary growth hormone (obtained by courtesy of Armour Laboratories).

Discussion. It is uncertain whether the enzyme detected by its action on GBNA is identical or not with one of the peptidases already described in serum. It seems to be closest to the enzyme hydrolyzing glycyl-leucine which, however, is activated by Zn(13). The relationship of GBNA peptidase with metallic ions remains unknown; its activation by Co, observed in human serum(2), was not confirmed in guinea pig serum. It seems that, if it requires any ion at all,—as is suggested by EDTA inhibition,—this has to be firmly

TABLE III. GBNA Hydrolysis and Plasmin Inhibition by Sera from Different Species.

Species	μM GBNA hydrolyzed/ml in 30 min.	Units plasmin inhibited/ml
Mouse	.030	40
Rat	.036	44*
Human	.038	51
Dog	.041	47
Rabbit	.081	36
Normal guinea pig	.143	66*
Cortisone-treated guinea pig	.194	85*
Growth hormone-treated guinea pig	.082	31*

* Results published in a previous study(3).

bound to the protein since it cannot be removed by dialysis.

Inhibitory action of certain biologically important amines (serotonin, histamine) is unlikely to have any physiological significance because they can hardly occur at the required concentrations.

On the basis of present data it cannot be decided whether GBNA-hydrolyzing enzyme is a true peptidase or a proteinase whose specificity is satisfied by the structure of the synthetic substrate. To inactivate trypsin or plasmin, it has to hydrolyze off their active groups. Since the nature and length of these groups is unknown we cannot even speculate on site of action of the enzyme.

The fibrinolytic system and other similar systems present in tissues appear to play a role in certain physiological and pathological processes(14). Rate of protease inhibition is an important factor controlling extent and duration of the processes. If the interpretation of present results is confirmed, they may supply an interesting example of one enzyme controlling the activity of another.

Summary. A peptidase, detectable by its action on chromogenic substrate glycyl-beta-naphthylamine, is present in serum of most species. In guinea pig serum, hydrolysis of GBNA and protease inhibition show a marked parallelism: they have a similar kinetic behavior, they are inhibited by the same agents and are under the influence of the same hormones. Competitive inhibition of peptidase action by plasmin and trypsin suggest that proteases are substrates for peptidase and that antiproteolytic activity of guinea pig serum, is due, partly at least, to enzymatic destruction of proteases. In some other species this mechanism may be less important.

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