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Effects of Some Enzyme Inhibitors on Fructosidase and Amylase

TAGE ASTRUP and WALBORG THORSELL

Biological Institute, Carlsberg Foundation, Copenhagen, Denmark, and Chemical Department, Veterinary College, Stockholm, Sweden

The effect of some protease nhibitors on the activity of fructosidase and amylase was studied. Generally speaking acid compounds were inhibitory in acid reactions, while basic compounds inhibited in alkaline reactions. The reactions were reversible and suggested a salt formation between enzyme and inhibitor. Protease inhibitors of protein type were generally inactive.

The effects of a number of enzyme inhibitors on the fibrinolytic activity of some proteases have been recorded recently[1,2]. Great differences were observed between the effects of the different protease inhibitors on the fibrinolytic activity of various proteases and the results could be used in the characterization of the different enzymes. It was of interest to study the effects of the inhibitory substances used in these investigations on some non-proteolytic enzymes. Two enzymes acting on uncharged carbohydrates were chosen for this preliminary investigation. They were β-h-fructosidase, acting on a low molecular weight carbohydrate, and salivary amylase, acting on a high molecular weight carbohydrate. These two enzymes were available as purified preparations.

MATERIAL

The following enzyme inhibitors were used: *1. Acids:* D,L-α-tocopherylphosphate (Hoffman-La Roche & Co. Ltd., Basel); laurylsulphate (sodium salt with 30 % sodium sulphate from Lundbeck & Co., Copenhagen); heparin (Løvens kemiske Fabrik, Copenhagen); chitin disulphuric acid and cellulose trisulphuric acid (prepared as previously described[3]). *2. Bases:* Cetylpyridinium chloride (Bionova Ltd., Copenhagen); toluidine blue and janus green (Grübler & Co. Ltd., Leipzig); laurylamine (Ferro Metal & Chem. Corp., New York). *3. Ampholytes:* Pulmin, the protease inhibitor isolated from ox lung[4] (a partially purified preparation was used[5]); soya bean trypsin inhibitor of Kunitz (about 70 % pure, prepared by a simplified method[6]); serum from cattle blood (a freeze dried preparation of spontaneously coagulated fresh blood). The substances were used as 2 % solutions in buffer with the exception of toluidene blue and janus green, which were used as 0.1 % solutions. The solutions were pH-corrected by addition of dilute sulphuric acid or sodium hydroxide and checked electrometrically.

Table 1. The effect of enzyme inhibitors on fructosidase. Enzyme activity expressed as change in rotatory power per min. ($\Delta\alpha/\Delta t$).

Inhibitor	$\Delta\alpha/\Delta t$			
	pH 3.5 ± 0.1 acetate	pH 4.5 ± 0.1 acetate	pH 6.5 ± 0.1 phosphate	pH 6.8 ± 0.1 citrate
None	0.15	0.15	0.13	0.08
D,L-α-Tocopherylphosphate	0.00	0.14	0.14	
Laurylsulphate	0.00	0.05	0.14	
Heparin	0.08	0.15	0.14	
Chitin disulphuric acid	0.06	0.15	0.13	
Cellulose trisulphuric acid	0.12	0.14		0.07
Cetylpyridinium chloride	0.14	0.14	0.05	
Toluidine blue	0.16	0.13	0.08	
Janus green	0.16	0.14		0.05
Laurylamine	0.14	0.16	0.10	0.07
Pulmin	0.09	0.14	0.12	
Soy bean inhibitor	0.13	0.15	0.13	
Serum	0.16	0.15	0.13	

Fructosidase was prepared from brewer's yeast and purified more than 1 000 times[7]. The amylase was prepared from human saliva and purified 5 times by precipitation with acetone[8]. The preparations contained no α-glucosidase.

EXPERIMENTS AND RESULTS

Effect on fructosidase: The experiments were performed in a water bath at 30.0° C and all solutions were preheated to this temperature and had identical pH values. The strength of the enzyme solution was chosen so that the reaction could be followed polarimetrically for 6 minutes[9].

6 ml fructosidase solution was mixed with 1 ml 0.2 *M* of an appropriate buffer solution (see the tables). 2 ml of an inhibitor solution was added to the mixture, followed after 30 secs. by 1 ml 0.2 *M* buffer solution. After a further 30 secs. 5 ml of the resulting mixture was added to a substrate solution consisting of 2.375 g saccharose dissolved in 38.3 ml dist. water and 5 ml 0.2 *M* buffer solution (saccharose concentration: 4.75 %). In some experiments the saccharose concentration was 9.50 % and in others 14.25 %. The hydrolysis was followed polarimetrically every minute for 6 minutes. From the straight line graph obtained the change in rotatory power per min. was calculated ($\Delta\alpha/\Delta t$). The values read were corrected for the small blank values of the solutions used. The results appear from Table 1.

As the activity optimum for the enzyme is pH 4.5 and the isoelectric point is at pH 5[10] it is seen that on the acid side (pH 3.5) very acid compounds only appear to inhibit, the reaction with cellulose trisulphuric acid being an excep-

Table 2. Reversibility of enzyme inhibition.

Inhibitor	$\Delta a/\Delta t$	
	pH 3.5 ± 0.1 acetate	pH 6.5 ± 0.1 phosphate
None	0.15	0.13
Heparin,	0.08	
» followed by cetylpyridinium chloride	0.13	
Chitin disulphuric acid,	0.06	
» » » followed by cetylpyridinium chloride	0.11	
Cetylpyridinium chloride,		0.05
» » followed by laurylsulphate		0.14

tion. Pulmin has a slight inhibitory action but too little is known about the chemistry of this compound to warrant any speculations. At pH 4.5 inhibition is less marked, occurring only with laurylsulphate. At pH 6.5 and 6.8 only alkaline substances act as inhibitors. Laurylamine has only slight effect.

Some of the inhibitors used in these experiments are surface active agents. They are known to have a denaturing effect on proteins, and it was thus of interest to find out whether the inhibition observed was caused by a simple denaturation of the enzyme, or if it was caused by a reversible combination with the inhibitor. Use was here made of the neutralization of surface active amines with surface active acids. These experiments were performed in principle as above but an appropriate concentration of the neutralizing agent was dissolved in the 1 ml 0.2 *M* buffer solution which was added after 30 secs. incubation of the enzyme inhibitor solution. Thus in one experiment (pH 6.5) the inhibition

*Table 3 *. Reversibility of enzyme inhibition.*

Inhibitor	$\Delta a/\Delta t$		
	Saccharose conc. %	pH 3.5 ± 0.1 acetate	pH 6.5 ± 0.1 phosphate
None	4.75	0.15	0.10
Heparin	4.75	0.05	
Chitin disulphuric acid	4.75	0.04	
Cetylpyridinium chloride	4.75		0.05
None	9.50		0.10
Cetylpyridinium chloride	9.50		0.04
None	14.25	0.13	0.10
Heparin	14.25	0.06	
Chitin disulphuric acid	14.25	0.05	
Cetylpyridinium chloride	14.25		0.06

* These experiments were performed with a fructosidase preparation purified about 200 times.

Table 4. Effect of enzyme inhibitors on amylase. Enzyme activity expressed as change in titration value per min ($\triangle ml/\triangle t$).

Inhibitor	$\triangle ml/\triangle t$					
	pH 5.9 ± 0.1		pH 6.9 ± 0.1		pH 7.9 ± 0.1	
	phos-phate	acetate	phos-phate	citrate	phos-phate	NH_3—NH_4Cl
None	0.20	0.20	0.25	0.31	0.17	0.18
D,L-α-Tocopherylphosphate	0.07	0.07	0.25		0.18	
Laurylsulphate	0.12		0.16		0.10	
Heparin	0.16		0.28		0.16	
Chitin disulphuric acid		0.20		0.30		0.17
Cellulose trisulphuric acid		0.21		0.29		0.17
Cetylpyridinium chloride	0.13		0.13		0.08	
Toluidine blue		0.16	0.19	0.23	0.14	0.13
Janus green		0.21	0.19	0.28	0.15	0.14
Laurylamine	0.18		0.19		0.15	0.17
Pulmin	0.21		0.28		0.15	
Soy bean inhibitor	0.21		0.26		0.15	
Serum		0.20	0.24		0.15	

with cetylpyridinium chloride was made reversible with 1 ml buffer solution containing 0.12 g laurylsulphate. In other experiments (pH 3.5) the inhibitory effect of heparin and chitin disulphuric acid could be reversed by the addition of 0.12 g cetylpyridinium chloride in 1 ml buffer. These results are summarized in Table 2.

The addition of an excess of substrate did not suffice to prevent the inhibition. This was the case when the substrate concentration was changed from the ordinary 4.75 to 9.50 and 14.25 %. Results of such experiments are presented in Table 3.

Effect on amylase: The experimental arrangement was as described above for fructosidase. 4 ml amylase solution was mixed with 2 ml 0.2 *M* buffer solution and incubated for 30 secs. with 2 ml inhibitor solution and 2 ml dist. water. 5 ml of the mixture was added to a substrate solution containing 25 ml fresh prepared 1 % solution of soluble starch (Baker's), 5 ml 0.2 *M* buffer solution and 1 ml 0.8 *M* sodium chloride. The enzymatic reaction was followed every minute for 6 mins. by the iodine method as described by Blom *et al.*[11] The change in ml 0.1 *N* thiosulphate solution per min. ($\triangle$ ml/$\triangle$ *t*) was constant and was calculated from the straight line graph obtained. Blank values were small and were used for correction. Table 4 shows the results.

On the acid side (pH 5.9) of the activity optimum (pH 6.9)[12] and near the isoelectric point (pH 5.1—5.9)[13] of the enzyme, inhibition was produced by tocopherylphosphate, laurylsulphate, (heparin) and cetylpyridinium chloride. With a neutral reaction (pH 6.9) inhibition was found with laurylsulphate and cetylpyridinium chloride. A slight inhibition was possibly also produced by the other bases tried. Very similar results were seen at pH 7.9.

DISCUSSION

These results deviate considerably from those obtained in the previous investigations of the inhibitory effects on proteases[1, 2]. Such a difference was expected as the nature of the enzymes and their substrates differed so much in the two series. The difference was apparent especially in the case of the ampholytes, which are very potent inhibitors of proteases, but which were without effect here, with the exception of pulmin, which had an inhibitory action on fructosidase in acid reaction (Table 1). It is also interesting that the polysaccharide sulphuric acid esters (heparin, chitin disulphuric acid, cellulose trisulphuric acid), which are known as potent inhibitors towards a number of proteolytic enzymes, were almost without effect in the systems here investigated. Only in very acid reaction (pH 3.5) with the fructosidase system was a pronounced effect observed. The acids were the most potent inhibitors in acid reactions. Similarly the bases were most active in the alkaline region. These results are in agreement with the inhibitory experiments of Myrbäck[14] and Quastel *et al.*[15] This may be an indication that the inhibition is caused by compound formation between the enzyme and the inhibitory substance by means of an ionic reaction. This is further strengthened by the velocity of the reaction, which is complete in a very short time. The reversibility of the inhibition on addition of an oppositely charged compound as seen in Table 2 is also evidence in these cases of salt formation between the enzyme and its inhibitor. A simple protein denaturation does not seem to occur under the conditions used here. — The interaction times were kept short in order to avoid secondary denaturing reactions. — When the reaction times were prolonged it was found that some denaturation of the enzyme could occur, because the decrease in activity could not be completely reversed by the addition of the appropriate neutralizing agent. Acid reaction and the surface active acids especially promoted this non-specific decrease in activity.

It was found that the inhibitory effect of the carbohydrate sulphuric acids, which may react with amino groups could be reversed by the addition of cetylpyridinium chloride (fructosidase pH 3.5). This was not so pronounced with an excess of substrate, though it was shown by Myrbäck[14] that an amino group in the enzyme is of importance in the enzyme substrate binding of fructosidase.

Though this investigation was of an orientating nature one may say that carbohydrate splitting enzymes, supposed to act on uncharged carbohydrate molecules, behave differently towards a number of inhibitory substances than is the case for proteases and protease inhibitors.

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