ACTA CHEMICA SCANDINAVICA 13 (1959) 1109–1112

The Action of Bacterial Trypsin on Tyrosine-Ethyl-Ester

S. O. ANDERSEN and JOHN GRAAE

Universitetets fysisk-kemiske institut, Copenhagen, Denmark

The splitting of tyrosine-ethyl-ester by a bacterial enzyme was studied. The reaction follows strictly the first order form to more than 99 % hydrolysis. pH optimum is found to 7.4 at 20°C and to 6.8 at 40°C. This variation can be explained by assuming that the enzyme attacks only the positively charged substrate molecules. The variation of activity with temperature is determined.

Bacterial trypsin is an enzyme isolated from *Bacillus subtilis*, closely related to, or identical with subtilisin. Subtilisin is mostly known for its proteolytic activity [1] but it has been shown that it is able to split methyl-esters of the lower fatty acids too [2,3]. In this work the hydrochloride of tyrosine-ethyl-ester is used as substrate, because it is a welldefined, reasonably soluble compound.

Crystalline bacterial trypsin was kindly placed at our disposal by *Novo Terapeutisk Laboratorium*, Copenhagen, Denmark.

The substrate was a commercial product (from Nutritional Biochemical Corporation) which was once recrystallized from ethyl-acetoacetate. Titration of the recrystallized product gave a molecular weight of 247.9 (Theoretical: 245.7).

The experiments were carried out in 50 ml N/10 KCl in which the desired amount of substrate was dissolved. Substrate concentrations varied from 8 mmoles/l to 20 mmoles/l. The enzyme was added and the hydrolysis followed by continuous automatic titration in a selfregistering pH-stat. If nothing else is stated temperature is 20°C and pH 7.0.

It was seen that the reaction strictly follows the first order form under all conditions examined. In Fig. 1 is plotted $\log_{10}(1-\alpha)$ *versus* t for an experiment followed to 99.7 % hydrolysis. For the routine determinations of the velocity constants a plot of log $(c_{t+\tau}-c_t)$ *versus* t was used, as suggested by Guggenheim [4]. $c_{t+\tau}$ is the amount hydrolysed at time $t+\tau$ and c_t is the amount hydrolysed at time t. The straight line obtained by this plot indicates that the reaction can be described by a first order form, with a velocity constant that is the slope of the line. It is experimentally proved that this constant, k', can be written as $k \cdot E$, where E is the enzyme concentration (see below).

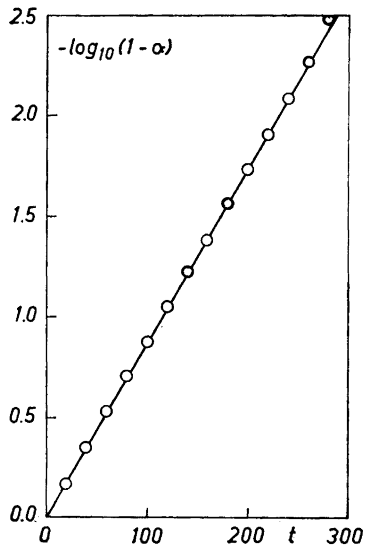

Fig. 1. As abscissa time in min and as ordinate $-\log_{10}(1-\alpha)$ for a typical experiment. Note that the degree of reaction at 260 and 280 min are 0.995 and 0.997, respectively. Temp. 20°C. pH = 7.0. Initial concentration of substrate = 17 mM. E = 8.75×10^{-4} mM. From the graph k is determined to 9 850 $min^{-1} M^{-1}$. At this experiment precipitation of tyrosine started at about 50 min. (α = 0.62.)

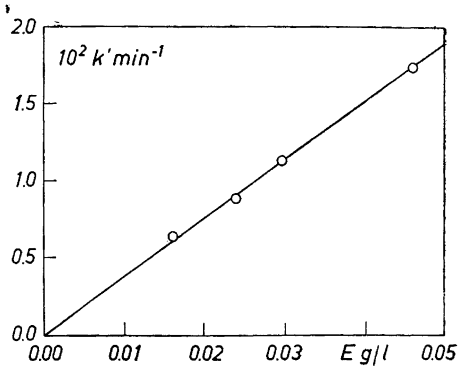

Fig. 2. As abscissa concentration of enzyme in g/l and as ordinate velocity constant k'. Temp. 20°C. pH = 7.0.

Where the constant is given per mole of enzyme it is based on a molecular weight of the enzyme of 27 500 [5]. The constant determined in this way is used for all further comparisons of experiments performed under different contions.

During the experiments it was noticed that after a period of supersaturation, tyrosine suddenly precipitated. As this precipitation in no way changed the fact that the reaction follows the first order form very strictly (*cf.* Fig. 1) it seems reasonable to conclude that the concentration of tyrosine has no influence at the reaction.

Table 1. The table gives the velocity constants determined with enzyme kept in solution in a refrigerator for the period indicated in the first column. pH = 7.0. Temp. = 20°C. Calculations based on $\log_{10}$.

Time, h	k min^{-1} l g^{-1}
0	0.396
4	0.374
23.6	0.392
26.2	0.398
47.4	0.382

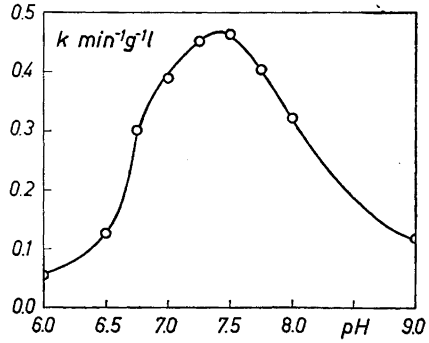

Fig. 3. Dependence of velocity constant on pH. Temp. 20°C.

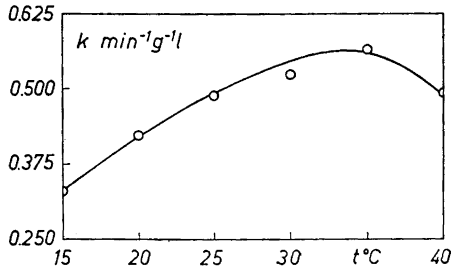

Fig. 4. Dependence of velocity constant on temperature. pH = 7.4.

As subtilisin is known to undergo selfdigestion when left alone, experiments were carried out in order to find out whether it was necessary to take special precautions to prevent selfdigestion. An enzyme sample was dissolved in water (1 g/l) and placed in a refrigerator. At suitable intervals an experiment was performed and the velocity constant determined. The results are given in Table 1. It is seen that there is no decrease in activity within 48 h.

The variation of the velocity constant with the enzyme concentration was studied. Experiments were performed at different enzyme concentrations and the constant determined in each case. From Fig. 2 it is seen that the constant is proportional to the enzyme concentration, as could be expected.

The variation of activity with pH was investigated. A pH optimum is found at 7.4 (Fig. 3). This is somewhat lower than that found when determined with other substrates [1-3].

The dependence of activity on temperature is shown in Fig. 4, where the experiments are performed at pH 7.4 at all temperatures. The pH was determined with a Sørensen phosphate buffer (80 ml M/15 Na_2HPO_4 and 20 ml M/15 KH_2PO_4) which is supposed to have pH 7.4 at all temperatures in the investigated interval. The somewhat strange decrease in activity at higher temperatures cannot be due to an irreversible denaturation of the enzyme, as the reaction still follows the first order scheme, and that indeed would not be expected if the enzyme were destroyed during the reaction. An investigation of the activity-pH curve has shown a shift in the pH-optimum with increasing temperature so that pH optimum at 40°C is lowered to pH = 6.9 (Table 2). It is most likely that this fact is the explanation of the peculiar form of the temperature-activity curve.

It is known that the pK values at 20°C of the amino group in amino acid esters is about 7—8 [6], and titration shows that the amino group in our sub-

Table 2. Variation of velocity constant with pH at 40°C.

pH	7.40	7.20	7.00	6.80	6.50
k min^{-1} l g^{-1}	0.495	0.571	0.665	0.659	0.576

strate has a pK value of 7.4 at 20°C and 6.95 at 40°C. This gives ΔH about 9 500 cal/mole in good agreement with findings for analogous compounds [6]. It therefore seems reansonable to conclude that the enzyme attacks only the positively charged substrate molecules, as this theory, combined with the found values for pK_{20} and pK_{40}, explains the finding of a pH optimum lower than that found ordinarily as well as the changing in pH optimum with temperature.

The pH optimum is usually found at about 8 [1-3] and it seems reasonable to assume that about this pH we have the highest concentration of the active form of the enzyme. But if the enzyme attacks only the positively charged tyrosine-ethyl-ester molecules this will give a lower pH optimum as the concentration of the positively charged molecules is very low at pH over and about 8. The effect of the temperature is understood in the same way as the pK for the substrate is lowered from 7.40 to 6.95 when the temperature is increased from 20°C to 40°C and therefore the concentration of the active — positively charged — form of the substrate is lowered at pH 7.4 (pH optimum at 20°C) and hence pH optimum is lowered.

REFERENCES

1. Ottesen, M. *Compt. rend. Lab. Carlsberg Ser. chim.* **30** (1958) 211 (Thesis).
2. Güntelberg, A. V. and Ottesen, M. *Compt. rend. Lab. Carlsberg Ser. chim.* **29** (1954) 36.
3. Graae, J. *Acta Chem. Scand.* **8** (1954) 356.
4. Guggenheim, E. A. *Phil. Mag.* **2** (1926) 538.
5. Marcker, K. *Personal communication.*
6. Cohn, E. J. and Edsall, J. T. *Proteins, Amino Acids and Peptides.* New York 1943.

Received April 9, 1959.