AN OPEN TUBULAR HETEROGENEOUS TRYPSIN REACTOR

J.TURKOVÁ[a], O.VALENTOVÁ[a], J.DOLEŽAL[b], J.ČOUPEK[c] and A.STOY[c]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6,*
[b] *Institute of Information Theory and of Automation, Czechoslovak Academy of Sciences, 180 76 Prague 8 and*
[c] *Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6*

Received April 17th, 1975

An open capillary reactor has been made of trypsin covalently attached to a polyacrylamide tube. The values of kinetic constants, $K_m = 4 . 10^{-3}$M and $V_{max} = 36$ nmol/s were determined in experiments with the cleavage of benzoyl-L-arginine ethyl ester as substrate. These values obtained graphically were confirmed by statistical treatment of the experimental data using the method of least squares and a computer.

During the past few years still more attention has been devoted to heterogeneous biocatalysts prepared by covalent attachment of enzymes to solid supports[1,2]. The catalysis of immobilized enzymes has been studied in enzyme reactors[3] of various types. It has been demonstrated that enzyme molecules bound to the surface of a solid support are utilized most in the catalysis whereas the molecules entrapped in the pores play a relatively small role[4]; therefore interest has shifted from the packed bed reactors and continuous stirred tank reactors to capillary through-flow reactors[5-9]. This paper reports on the construction of an open capillary trypsin reactor and on the determination of its kinetic constants on the basis of cleavage of benzoyl-L-arginine ethyl ester effected in the through-flow arrangement.

EXPERIMENTAL

Materials

Trypsin was a product of Léčiva, Prague. Benzoyl-L-arginine ethyl ester (BAEE) was from Schuchardt, München. The polyacrylamide support was prepared from the polymer solution in the form of a tube 2 mm in diameter by the extrusion technique and subsequent cross-linking. The support contained free carboxyl groups which were used for the reaction with the enzyme.

Binding of Trypsin to Gel Tubes

Trypsin was bound to the free carboxyl groups of the polyacrylamide tubes through a water-soluble carbodiimide. Trypsin (50 mg) was dissolved in 20 ml of 0.1M phosphate buffer at pH 6·0

and this solution was treated with 230 mg of N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride. The end of a 150 cm long tube was immersed into the solution, the remaining part of the tube was dipped into 0·1M phosphate buffer at pH 6·0. The other end of the tube was connected with a pump which transferred the reaction solution back to the first vessel (flow rate 1·1 ml/min). The enzyme solution was removed after 18 h and the tube was washed stepwise with distilled water, 0·1M sodium borate containing 1M-NaCl, pH 8·0, 0·1M sodium acetate containing 1M-NaCl, pH 4·1, 0·01M sodium. acetate pH 4·1, and lastly with 0·15M sodium borate containing 0·02M-$CaCl_2$, pH 7·5; the tube with bound trypsin was stored in the latter solution. All operations were carried out at 4°C. The buffer in which the tube was immersed during the reaction, showed no tryptic activity after the completion of the binding. The quantity of enzyme bound to 1 cm of tube was 0·27 mg.

Trypsin Reactor

The construction of the trypsin reactor is obvious from the scheme shown in Fig. 1. Thermostated vessel *A* accomodates the substrate reservoir marked *B*. The substrate solution is pumped by a peri-

TABLE I

Experimentally Determined Values of Product Concentration as Function of Substrate Concentration

Substrate concentration mM	Absorbance	Product concentration	
		nmol/ml	nmol/s
0·5	0·250	215	3·95
1·0	0·435	375	6·9
1·25	0·560	485	8·9
2·5	0·900	782	14·35
5·0	5·700	4 950	90·8

TABLE II

Basic Data for Statistical Treatment of Kinetic Constants

[*S*] mM	*v* nmol/s	Relative weights		
		from (*11*)	from (*12*)	from (*13*)
0·40	3·942	0·217	0·431	0·073
1·00	6·875	0·149	0·151	0·294
1·25	8·892	0·412	0·338	0·300
2·50	14·337	0·137	0·059	0·193
5·00	90·750	0·085	0·021	0·140

TABLE III

Values of Kinetic Constants and Basic Statistical Characteristics of Population of Experimental Data Measured

Weights choice	Run	K_m mM	V_{max} nmol/s	$t_{0,05}$ tabul.	t calc.	r_{xy}
Unweighed case	1	21·4 ± 46·7	168 ± 353	4·30	7·07	0·9883
	2	4·75 ± 1·19	41·2 ± 8·8	12·71	3·09	0·9986
Weights from (*11*)	1	9·67 ± 8·73	78·8 ± 65·9	4·30	4·33	0·9915
	2	5·20 ± 1·40	44·9 ± 10·5	12·71	2·65	0·9986
Weights from (*12*)	1	6·08 ± 2·69	51·7 ± 20·7	4·30	2·19	0·9969
	2	—	—	—	—	—
Weights from (*13*)	1	20·4 ± 42·9	155 ± 310	4·30	4·89	0·9826
	2	5·10 ± 1·81	43·5 ± 13·2	12·71	3·71	0·9962

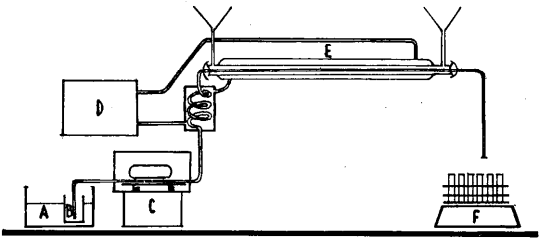

FIG. 1

Scheme of Through-flow Capillary Reactor

A Thermostat 1, B reservoir of substrate solution, C pump, D thermostat 2, E through-flow reactor, F fraction collector.

staltic pump *via* a heated capillary coil to jacketed column E. The heated jacket accomodates a glass tube filled with silicone oil. The tube is connected with two funnels which keep the oil level constant. The oil-containing tube is closed at both ends with ball joints. The latter are provided with metal nipples to which the gel capillary (1 m long) with trypsin bound to its inner surface is attached. Silicone oil is used to prevent the low molecular weight substrate from diffusion out of the reactor. Solutions of BAEE at various concentrations in 0·1M phosphate buffer, pH 8·0, were used as substrates. The substrate was cleaved to benzoyl-L-arginine and ethanol during passage through the column. The mixture of product and substrate was collected in automatic fraction collector F at a rate of 5·5 mol/5 min. The quantity of benzoyl-L-arginine in the fractions was determined by absorbance measurement at 253·6 nm according to Horvath and Solomon[5]. The value of the apparent K_m-constant was determined from the data measured (Table I) graphically according to Lineweaver and Burke (Fig. 3) and in an IBM 370/135 computer (Tables II and III).

RESULTS AND DISCUSSION

The quantity of benzoyl-L-arginine in the effluent from the reactor corresponding to the individual concentrations of BAEE was determined. As can be seen in Fig. 2, a steady state is attained after a certain time where the quantity of product formed per time unit is constant and temperature-dependent. The graphical Lineweaver-Burke plot of the apparent K_m-constant is shown in Fig. 3. The graphically determined values for bound trypsin are $K_{m\,app} = 4 . 10^{-3}$M and $V_{max} = 36$ nmol/s.

More exact and objective values yields the statistical treatment of the data measured by the method of least squares. In this case we deal with a linear regression of the type $y = a + bx$, where constants a and b are parameters of this regression; x and y correspond to $[S]^{-1}$ and v^{-1}, respectively. Here v denotes the steady-state reaction velocity at a given substrate concentration $[S]$. These parameters can be determined by well-known procedures (*e.g.*[10-12]).

Then

$$K_m = b/a \quad \text{and} \quad V_{max} = 1/a\,. \tag{1}$$

To estimate K_m and V_{max} we use equations (2) and (3)

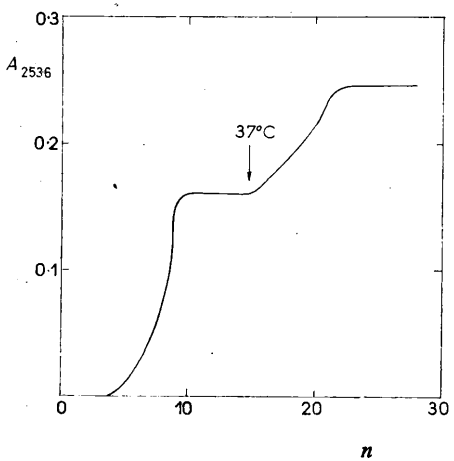

FIG. 2

Spectrophotometric Evaluation of Reaction Rate of Benzoyl-L-arginine Ethyl Ester Cleavage Catalyzed by Immobilized Trypsin

$A_{253.6}$, absorbance corresponding to concentration of benzoyl-L-arginine formed; n, order of fractions emerging from the enzyme reactor. The arrow marks the temperature increase from 25° to 37°C.

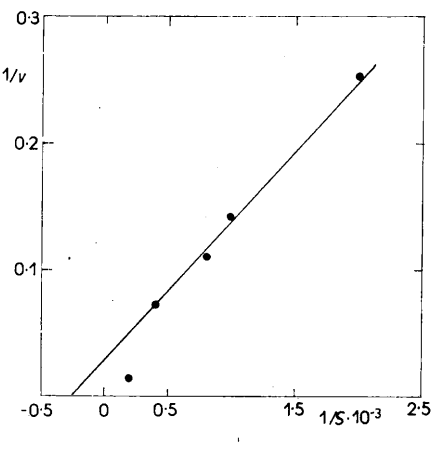

FIG. 3

Graphical Evaluation of Kinetic Constants of Esterolysis of Benzolyl-L-arginine Ethyl Ester Catalyzed by Immobilized Trypsin

v, Reaction rate expressed in nmol of benzoyl-L-arginine formed per s; S, substrate (concentration of benzoyl-L-arginine ethyl ester).

$$K_m = \frac{(\sum_i w_i)(\sum_i w_i v_i^{-1}[S_i]^{-1}) - (\sum_i w_i [S_i]^{-1})(\sum_i w_i v_i^{-1})}{(\sum_i w_i [S_i]^{-2})(\sum_i w_i v_i^{-1}) - (\sum_i w_i [S_i]^{-1})(\sum_i w_i v_i^{-1}[S_i]^{-1})}, \quad (2)$$

$$V_{max} = \frac{(\sum_i w_i)(\sum_i w_i [S_i]^{-2}) - (\sum_i w_i [S_i]^{-1})^2}{(\sum_i w_i [S_i]^{-2})(\sum_i w_i v_i^{-1}) - (\sum_i w_i [S_i]^{-1})(\sum_i w_i v_i^{-1}[S_i]^{-1})}, \quad (3)$$

where adding index i must be considered within limits 1 to N (N designates the number of points (pairs) measured ($[S_i]$, v_i) and w_i, $i = 1,\ldots,$ N is the weight of the i-th point). The equations given are valid on condition that concentration $[S]$ has been measured without error and that random variables which affect the individual reaction rates v show the same zero mean value, are not correlated, and all show the same dispersion.

The number of all points measured N is usually not too high (less than ten). This follows from the practical demands on the individual measurements. In spite of that, however, a statistical treatment of even small populations is useful since thus the data measured supply more information on the process investigated.

The variance of the parameters of linear regression can be estimated from

$$s_a^2 = s_{xy}^2 \frac{\sum_i w_i [S_i]^{-2}}{(\sum_i w_i)(\sum_i w_i [S_i]^{-2}) - (\sum_i w_i [S_i]^{-1})^2}, \quad (4)$$

$$s_b^2 = s_{xy}^2 \frac{\sum_i w_i}{(\sum_i w_i)(\sum_i w_i [S_i]^{-2}) - (\sum_i w_i [S_i]^{-1})^2}, \quad (5)$$

$$s_{xy}^2 = \frac{1}{N-2}\left\{\sum_i w_i v_i^{-2} - \frac{(\sum_i w_i v_i^{-1})^2}{\sum_i w_i} - \right.$$

$$\left. - \frac{[(\sum_i w_i)(\sum_i w_i v_i^{-1}[S_i]^{-1}) - (\sum_i w_i v_i^{-1})(\sum_i w_i [S_i]^{-1})]^2}{(\sum_i w_i)[(\sum_i w_i)(\sum_i w_i [S_i]^{-2}) - (\sum_i w_i [S_i]^{-1})^2]}\right\}. \quad (6)$$

The variance of the value of maximum reaction rate s_V^2 can be estimated from

$$s_V^2 = \left(\frac{\partial V_{max}}{\partial a}\right)^2 s_a^2 = \frac{s_a^2}{a^4} = V_{max}^4 s_a^2 . \quad (7)$$

Parameters a and b are not stochastically independent. Hence, the variance of the value of Michaelis constant s_K^2 must be estimated from

$$s_K^2 = \left(\frac{\partial K_m}{\partial a}\right)^2 S_a^2 + \left(\frac{\partial K_m}{\partial b}\right)^2 s_b^2 + 2\frac{\partial K_m}{\partial a}\frac{\partial K_m}{\partial b}\operatorname{cov}(a, b) =$$

$$= \frac{b^2}{a^4} s_a^2 + \frac{s_b^2}{a^2} + \frac{2bs_{xy}^2(\sum_i w_i[S_i]^{-1})}{a^3[(\sum_i w_i)(\sum_i w_i[S_i]^{-2}) - (\sum_i w_i[S_i]^{-1})^2]} =$$

$$= V_{max}^2 s_{xy}^2 \frac{K_m^2(\sum_i w_i[S_i]^{-2}) + 2K_m(\sum_i w_i[S_i]^{-1}) + \sum_i w_i}{(\sum_i w_i)(\sum_i w_i[S_i]^{-2}) - (\sum_i w_i[S_i]^{-1})^2}. \quad (8)$$

The standard errors of K_m and V_{max} are then represented by the square roots of the corresponding variance estimates made according to equations (7) and (8).

To obtain complete information, it is desirable to determine the magnitude of the so-called correlation coefficient r_{xy} given by

$$r_{xy} = \frac{(\sum_i w_i)(\sum_i w_i v_i^{-1}[S_i]^{-1}) - (\sum_i w_i[S_i]^{-1})(\sum_i w_i v_i^{-1})}{\sqrt{\{[(\sum_i w_i)(\sum_i w_i[S_i]^{-2}) - (\sum_i w_i[S_i]^{-1})^2][(\sum_i w_i)(\sum_i w_i v_i^{-2}) - (\sum_i w_i v_i^{-1})^2]\}}}. \quad (9)$$

This coefficient is a measure of the linear dependence of $[S]^{-1}$ and v^{-1}.

For most populations treated as described it is necessary to determine whether the deviations of some of the data measured from the regression line calculated do not substantially differ from normal distribution. This can be achieved by the Student test of significance in a manner analogous to the procedure described elsewhere[13]. It consists of seven steps: *1.* We determine the regression line (parameters a, b) from all the data measured. *2.* We calculate reaction rates $\tilde{v}_i$ from $\tilde{v}_i = 1/(a + b[S_i]^{-1})$ for all the concentrations $[S_i]$ used. *3.* We determine the point where the absolute value of weighed deviation, *i.e.* $|\sqrt{w_i}[(v_i)^{-1} - (\tilde{v}_i)^{-1}]|$ is maximum. *4.* We do not consider in further calculations data corresponding to this point, *i.e.* values ($[S_m]$, v_m, w_m), and determine a new regression line (parameters a^*, b^*) from the remaining data. *5.* We determine reaction rate $v_m^* = 1/(a^* + b^*[S_m]^{-1})$ corresponding to concentration $[S_m]$ using the new regression curve and determine the corrected value of maximum weighed deviation, *i.e.* $\sqrt{w_m}[(v_m)^{-1} - (v_m^*)^{-1}]$. We calculate standard error s_{xy}^* for the new regression line from equation (6). *6.* The significance of the corrected maximum weighed deviation from the new regression curve is established by the Student test. For this purpose we determine

$$t = \left| \sqrt{w_m} \frac{[(v_m)^{-1} - (\tilde{v}_m^*)^{-1}]}{s_{xy}^*} \right| . \qquad (10)$$

7. The t-value thus obtained is then compared with tabulated values of $t_\alpha(N - 3)$ (ref.[11]), where α stands for the significance level (risk) given or chosen; we usually choose $\alpha = 0{\cdot}05$. If t does not exceed the tabulated values, $t \leqq t_\alpha(N - 3)$, the data ($[S_m]$, v_m, w_m) are statistically significant and the values of K_m and V_{max} can be determined from (*1*) using the first regression curve and parameters a, b. Standard errors s_K, s_V and correlation coefficient r_{xy} are calculated from equations (*7*)–(*9*). In the opposite case we do not consider these data further and repeat the whole procedure with a reduced number of data until the Student significance test is either fulfilled or only three points remain.

An acceptable procedure appears to be a weight choice with the aid of absolute, relative, or standard errors of the individual points with respect to the regression line determined from all data. In the first case we choose weights numerically equal to the relations*

$$w_j = \frac{1}{|(v_j)^{-1} - (\tilde{v}_j)^{-1}|}, \quad j = 1, \ldots, N, \qquad (11)$$

in the second case

$$w_j = \left| \frac{(\tilde{v}_j)^{-1}}{(v_j)^{-1} - (\tilde{v}_j)^{-1}} \right|, \quad j = 1, \ldots, N, \qquad (12)$$

and finally in the third case

$$w_j = \frac{N(\sum_i [S_i]^{-2}) - (\sum_i [S_i]^{-1})^2}{\tilde{s}_{xy}^2 [N[S_j]^{-2} - 2[S_j]^{-1} (\sum_i [S_i]^{-1}) + \sum_i [S_i]^{-2}]}, \quad j = 1, \ldots, N. \qquad (13)$$

The last method of weight choice from equation (*13*), *i.e.* where the weights are given by reciprocal values of variance estimate of the corresponding point, is sometimes recommended as being statistically effective. However, sometimes even the unweighed version of the procedure described will prove sufficient; then $w_i = 1$, $i = 1, \ldots, N$.

The determination of kinetic constants is very common in chemical practice. For this reason the procedure described here has been programmed in language PL/1 and verified on a computer. It has been tested with several populations of data taken

* The values of $\tilde{v}_j$, $j = 1, \ldots, N$ and $\tilde{s}_{xy}$ are determined as in Step 2 using a preliminary unweighted regression.

from papers dealing with this subject (*e.g.*[9,13]). The results obtained by us were always identical with the conclusions made in these studies. Moreover, our calculation was more accurate than the graphical procedures used; we were also able to obtain the basic characteristics of the populations examined. All the calculations described here required on the average roughly three seconds of computer time per example examined.

We have treated also the population of data measured with us by this procedure. The basic values are given in Table II and the results obtained are treated summarily in Table III. Except for the case of weights according to equation (*12*), it was always the last point (Table II) which in the first run of the Student significance test was found to be insignificant, this fact being in agreement with the graphical investigation since this point considerably deviates from the theoretical linear dependence given by the remaining points. This point was therefore neglected in the second run. All the remaining points were found to be significant (Table III).

It also follows from Table III that the results obtained by all four procedures of weight choice are essentially equivalent and in agreement with the above given values of kinetic constants obtained by the graphical procedure. The situation with the remaining examples solved was similar and it is therefore difficult to recommend the most convenient method of weight choice. It should be also noted that by the neglection of the insignificant point the estimates of the standard errors were stepwise reduced to an acceptable degree. Likewise the correlation coefficient increased after the neglection of the insignificant point.

REFERENCES

1. Goldman R., Goldstein L., Katchalski E. in the book: *Biochemical Aspects of Reactions on Solid Supports* (G. R. Stark, Ed.). Academic Press, New York 1971.
2. Turková J.: Chem. Listy *68*, 489 (1974).
3. O'Neill S. P.: Rev. Pure Appl. Chem. *22*, 133 (1972).
4. Lilly M. D., Sharp A. K.: Chem. Eng. (New York) *215*, CE 12 (1968).
5. Horvath C., Solomon B. A.: Biotechnol. Bioeng. *14*, 885 (1972).
6. Horvath C., Shendalman L. H., Light R. T.: Chem. Eng. Sci. *28*, 375 (1973).
7. Horvath C., Sardi A., Woods J. S.: J. Appl. Physiol., *34*, 181 (1973).
8. Kobayashi T., Laidler K. J.: Biotechnol. Bioeng. *16*, 99 (1974).
9. Bunting P. S., Laidler K. J.: Biotechnol. Bioeng. *16*, 119 (1974).
10. Lineweaver H., Burk J.: J. Amer. Chem. Soc. *56*, 658 (1934).
11. Zar J. H.: *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, N. J. 1974.
12. Rektorys K.: *Přehled užité matematiky*. Published by SNTL, Prague 1963.
13. Elmore D. T., Kingston A. E., Shields D. B.: J. Chem. Soc. *II*, 2070 (1963).

Translated by V. Kostka.