

Alkaline Phosphatase from *Walterinnesia Aegyptia* (Desert Cobra) Venom: Optimization and Kinetic Properties

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Abstract. Alkaline phosphatase (ALP) was investigated and characterized with respect to its kinetic properties in the venom of *Walterinnesia aegyptia*. It was observed that 50 µg of crude venom protein was suitable for linearity of ALP activity at 25°C. The optimum pH, temperature and strength of diethanolamine were found to be 9.8, 40°C and 0.9 M respectively. The heat of activation and energy of activation were found to be 5663 Cal/mol and 6253 Cal/mol respectively. The optimum substrate concentration was observed to be 8mM. The *p*-nitrophenyl phosphate (PNPP) concentration ranges for different orders of the reactions were also determined. The various kinetic parameters (K_m , V_{max} , K_{cat} and the ratio of K_{cat}/K_m) were established at each order of the reaction.

Key words : Venom, alkaline phosphatase, kinetics, optimization, turnover number.

Introduction

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase; ALP, EC 3.1.3.1) is widely distributed in nature. There are considerable species and organ-related differences; even within the same organ and the same cell, enzymatic activity is likely to be unevenly distributed (McComb et al; 1979a). A number of researchers (Gullard, 1938, Zeller, 1950; Yang and Chan 1954; Kaye, 1960, Richards *et al.*, 1965; Jimenez-Porras, 1964a, Zeller 1951) have reported the presence of phosphatases in snake venoms (Tu and Chua; 1966). Phospho-monoesterase is found in the venom of Elapidae, Hydrophidae, Viperidae and Crotalidae. Uzawa (1932) was the first to report the presence of phosphomonoesterase in the venom of *Trimeresurus flavoviridis* (Tu; 1977). It is suggested that the action of snake

venom phosphatases is related to shock symptoms (Zeller; 1951). Tu and Chua (1966) reported the presence of alkaline phosphatase in five members of Elapidae which all belonged to genus *naja*. Nonspecific alkaline phosphatase from *Bothrops atrox* venom has been purified, characterized and studied in detail (Sulkowski *et al*; 1983). Hassan *et al*; 1981 studied in detail the acid and alkaline phosphatases in two members of Elapidae; *Naja haje* and *naja nigricollis*. In the present study, we show that alkaline phosphatase is present in the venom of the desert cobra (*Walterinnesia aegyptia*) a member of the elapidae which has not been previously investigated probably because of its rarity. In Saudi Arabia, however, the *W. aegyptia* is a major problem, because it is distributed widely and its bites are frequently fatal (Al-Sadoon; 1989). In a previous work,

we have reported that the venom of *W. aegyptia* is a complex mixture of seven protein fractions. Fraction 5 exhibited lethal effect (Al-Saleh *et al*; 1994).

Materials and Methods

The substrate *p*-nitrophenylphosphate (pNPP) was purchased from Amresco SOLON, OHIO, USA. Diethanolamine (DEA) was obtained from Fluka Chemie AG, Buchs, Switzerland. Magnesium sulfate was from Riedel-dekHen AG, Wunstorfer StraBe, Germany. All other chemicals used were of analytical grade.

W. aegyptia were captured in the desert of Saudi Arabia. They were kept in animal house, maintained at $28^{\circ} \pm 2^{\circ}\text{C}$ and with a diurnal cycle system of 17h dark and 7h light. They were fed with 1 mouse (20-30 g) per snake every two weeks and water ad libitum.

Venom from two desert cobras (60 mg of protein/200 μl of venom fluid) was collected and dialyzed overnight in 50mM sodium acetate buffer pH 5.8 containing 500 mM NaCl. The venom was centrifuged at 5,000 g for 15 minutes to remove any turbidity and before use diluted with the appropriate buffer as mentioned in results and discussion section.

The ALP activity was measured according to the spectro-photometric method of Hausamen *et al*; 1967 at 25°C . The assay mixture (1.2 ml) contained 0.9 M diethanolamine (DEA) buffer pH 9.8, 8mM *p*-nitrophenyl phosphate, 0.5m M magnesium sulphate and 5-50 μg venom protein as the source of the enzyme. One unit of enzyme activity is defined as the amount of the ALP that catalyzes the hydrolysis of 1 μmole of PNPP per min per mg of venom protein under

standard conditions.

The energy of activation (E_a) for the hydrolysis of pNPP by *W. aegyptia* venom ALP was determined by plotting the log of ALP activity Vs $1/T$ (Arrhenius plot). The value of the negative slop of this plot was put in to the following equation to give the energy of activation,

$$E_a \quad \text{Slop} = \frac{E_a}{2.3R} \quad \text{-----} \quad (\text{Segel; 1975a})$$

whereas R is the Boltzman constant and its value is equal to 1.98.

The heat of activation (ΔH^*) was calculated by the following equation:

$$E_a = \Delta H^* + RT$$

(E_a is energy of activation)

The temperature coefficient, Q_{10} (which is the factor by which the rate constant is increased by raising the temperature by 10°C) was calculated by the following equation:

$$E_a = \frac{2.3R \times T_1 \times T_2 \times \log Q_{10}}{10}$$

(where T_1 and T_2 are the two temperatures)

The turnover number of the ALP, K_{cat} , is defined as the maximum number of the moles of pNPP that were converted to product each min per mole of ALP. The equation, $K_{cat} = V_{max}/(ET)$ was used to calculate turnover number, where (ET) is the total enzyme concentration, and standard graphical method was used to determine (ET) (Dixon; 1972). The ratio of K_{cat}/K_m was also calculated.

The protein content of the enzyme preparation was determined by the method of Lowry *et al*; 1951.

Results and Discussion

The enzyme activity was measured at different concentration of venom protein (5-60 µg of protein) and found to be linear from 5-50 µg of venom protein. The activity being maximum at 50µg of venom protein. Therefore this amount was selected for further studies of ALP (Fig 1).

ALP activity was determined at various pH values ranging from 6.0–12.0 (Fig 2). The enzyme activity was linear from pH 8.5–9.8, being maximum at pH 9.8. So pH 9.8 was selected as optimum pH for further studies. It had been documented by Tu and Chua ; 1966 that ALP of five snake venoms from Elapidae has optimum pH 9.0. Hassan *et al*; 1981 reported that ALP from 2 snake venoms of viperidae have optimum pH 10.0 and ALP from 2 snake venoms of Elapidae have optimum pH 9.0. It is mentioned by Sulkowski *et al*; 1983 that Bothrops atrox (crotolidae) ALP has a pH optimum in the neighborhood of 9.5. It is possible that this really reflects a species different, but discrepancy might also due to different degrees of purification of enzyme or to different ionic media used in the enzyme assay.

ALP activity was measured in the presence of different strengths of DEA buffer (0.1–2.0 M), pH 9.8. ALP activity was maximum at 0.9M (Table 1), therefore, it has been considered as 100% relative specific activity for comparing with the activity of other buffer strengths. As indicated in Table 1, that the ALP activity is increasing with the increase in buffer strength. This is due to the fact that alcoholic compounds increase the ALP activity with increasing concentration which is presumably due to an increase in trans-phosphorylation. It is also reported that

Table 1. Effect of DEA buffer, strength on *W. aegypti* Venom alkaline phosphatase.

Buffer strength (M)	Specific activity (µmole/min/mg protein)	Activity (%)
0.1	16.5	40.0
0.2	27.5	66.6
0.3	30.0	72.6
0.4	30.3	73.4
0.5	35.8	86.7
0.6	35.8	86.7
0.7	38.8	93.2
0.8	38.8	93.2
0.9	41.3	100.0
1.0	30.3	73.4
2.0	30.0	72.6

Each value represents mean of three independent experiments. the rate of phosphate transfer was a function of the type and concentration of the acceptor alcohol but was independent of free energy of the phosphate bond of the donor (substrate). It is indicated in [Table 1] that a decrease of 26.6–27.4% ALP activity has been observed at a range of 1.0–2.0 M buffer strength. It is reported that moderate concentrations of certain amino-alcohol acceptors markedly stimulate total ALP activity but at high concentration (2.0 M/L) even powerful trans-phosphorylators tend to become inhibitory (McComb *et al*; 1979b). It was also reported that buffer strength plays an important role in regulating enzyme activity, through its effect on protein conformation in general (Dawson and Crone; 1973).

The ALP activity of the crude venom at different temperature (10–60°C) was also determined. There was a proportional increase from 20–40°C with maximum activity at 40°C (Fig.3). The ALP of crude venom became inactivated at 55°C. It is mentioned by Hassan

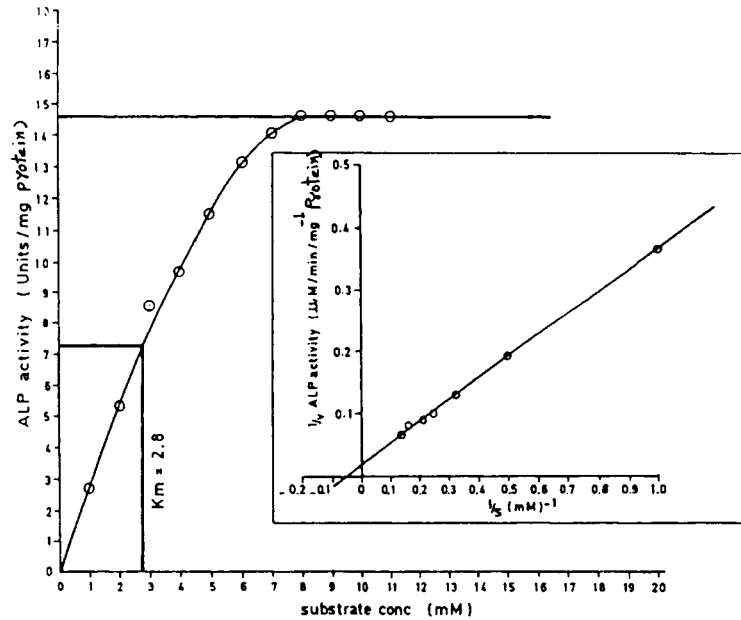


Fig. 1. Effect of substrate concentration on the rate of hydrolysis of *P*-nitrophenyl phosphate by *W. aegyptia* venom alkaline phosphate. Plot is specific activity against the concentration. Each point represents the means of four independent experiments. (inset) Lineweaver Burk plot for the hydrolysis of the substrate (PNPP) by *W. aegyptia* ALP at a wide concentration range (0.1-100 Mm) for preliminary estimation of the K_m and V_{max} values. Each point represents the mean of four independent experiments.

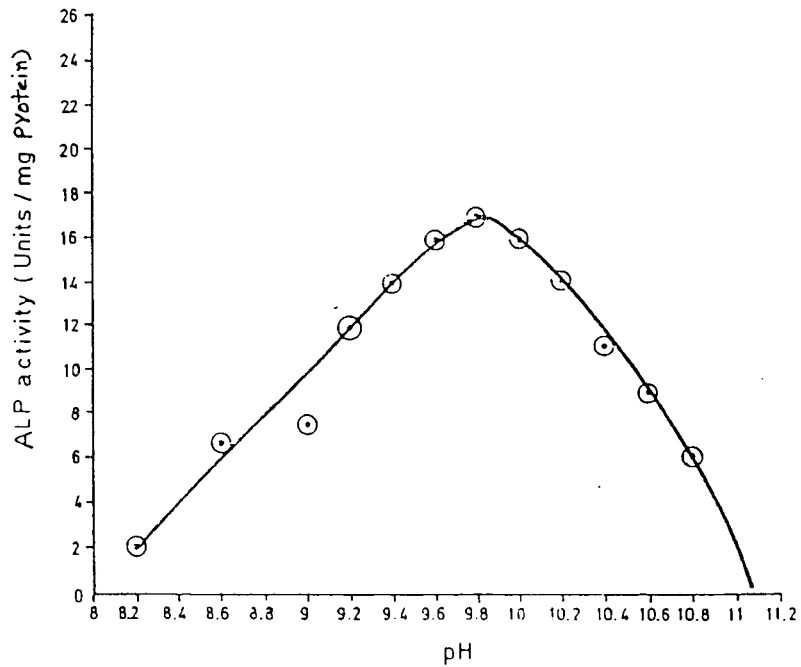


Fig. 2. Effect of pH on *W. aegyptia* alkaline phosphatase activity at 25°C. Each point represents the mean of three independent experiments.

et al; 1981 that the ALP activity of two Cerastes and 2 Naja was linear from 37°C–40°C and beyond this range deterioration in the enzyme activity occurred. Setogouchi *et al* [6] showed that the phosphatases of *L. semifasciata* were heat labile at 57°C. Yora *et al*; 1986 found that snake venom phosphatases are highly sensitive to heat treatment when heated at 56°C for 5 minutes. The ALP from *Bothrops atrox* venom was also found to be unstable at 55°C (Sulkowski *et al*; 1983).

The energy of activation (Ea) for ALP was determined by using the slope (-1373) of Arrhenius plot for enzyme. It was estimated as 6253 Cal/mol. The H* was calculated as 5663 Cal/mol at 25°C and Q10 was 1.44. It is reported by McComb *et al*; 1979 that the Ea is 6880 Cal/mol for *E. coli* ALP; 10,400 Cal/mol for human placental ALP, 10,000 Cal/mol for human and feline skeletal ALP (β -glycerophosphate as substrate) and 6000 cal/mol for rat intestinal enzyme. Asgeirsson *et al*; 1995 have documented energy of activation (Ea) 20.9±2.10 KJ/mol and 33.0±2.76 KJ/mol for Atlantic Cod and Calf intestinal ALP respectively. They also reported the values of free energy of activation (G*) which are 51.38 KJ/mol for cod and 52.41 KJ/mol for calf respectively.

In order to recognize the different orders of reaction, the effect of different concentration of substrate was also studied. The range of the final PNPP concentration in 1 ml assay media was 0.1–100 mM. A linear increase of velocity was observed from 0.1–7.0 mM (Fig.1). The optimum substrate concentration for maximum ALP activity was calculated from Fig.1 as 8.0 mM. Hassan *et al*; 1981 reported 100 mM for *Cerastes cerastes* and *Cerastes vipera* and 150 mM for *Naja haje* and 125 mM for *Naja*

Table 2. Characterization of p-nitrophenyl phosphate Hydrolysis reaction by *W. aegyptia* venom Alkaline phosphatase

Reaction Order	K _m (mM)	V _{max} U/mg	10 ⁻³ xK _{cat} (Sec ⁻¹)	10 ⁻³ xK _{cat} /K _m Sec ⁻¹ mM ⁻¹
First order	0.33	0.46	4.2	12.7
Pseudo-1 st Order	2.2	1.15	4.9	2.24
Zero-order	3.5	1.5	4.0	1.16

nigricollis. The large difference among the optimum substrate concentration may be due to different assay methods used for the activity determination. The higher concentration of PNPP (13.0–100.0 mM) inhibited the activity of enzyme. It was observed that 100 mM PNPP inhibited the enzyme by 84 as compared to the activity at 8mM PNPP. The substrate inhibition constant (K_{si}) was calculated as 0.022 M (method of Dixon and Webb; 1979). K_m and V_{max} were measured over wide range of PNPP concentrations (Fig.1) and found to be 2.8 mM and 13.75µM/Min/mg respectively. Only a preliminary estimation of K_m and V_{max} was obtained at these PNPP concentrations. The data is used to select three other different ranges of PNPP concentration for determination of precise K_m and V_{max} values at different orders of reactions i.e. first order, pseudo first order or optimal order and zero order kinetics (Segel; 1975b).

It is found that the hydrolysis of PNPP by *W. aegyptia* ALP is first order in concentration range 0.08–0.5 mM (Fig. 4A); Pseudo-first order in the range 0.8–5 mM (Fig. 4B) and zero order for the range 8–50 mM (Fig. 4C). The slope of straight line in Fig. 4C was lower than the slopes of lines Fig. 4A & B because the PNPP concentration was higher than the

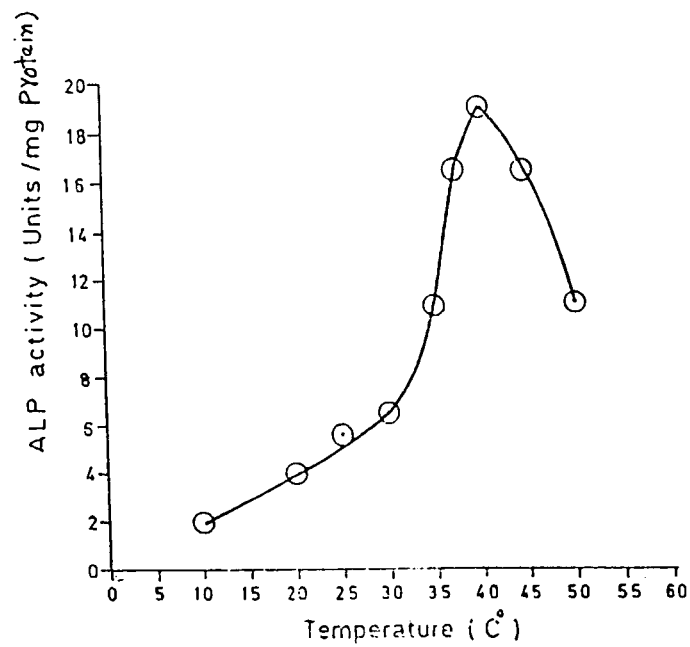


Fig. 3. Effect of temperature on *W. aegyptia* alkaline phosphatase at 8 mM PNPP and pH 9.8. Each point represents the mean of five values.

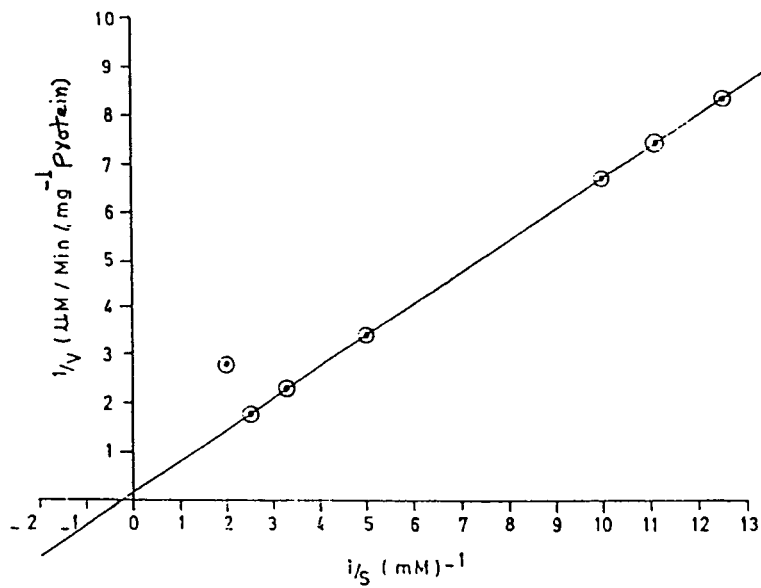


Fig. 4A. Lineweaver-Burk plot for the hydrolysis of p-nitrophenyl phosphate by *W. aegyptia* alkaline phosphatase at: (A) the lowest substrate concentration (0.08-0.5 mM), for almost first-order reaction with respect to [PNPP].

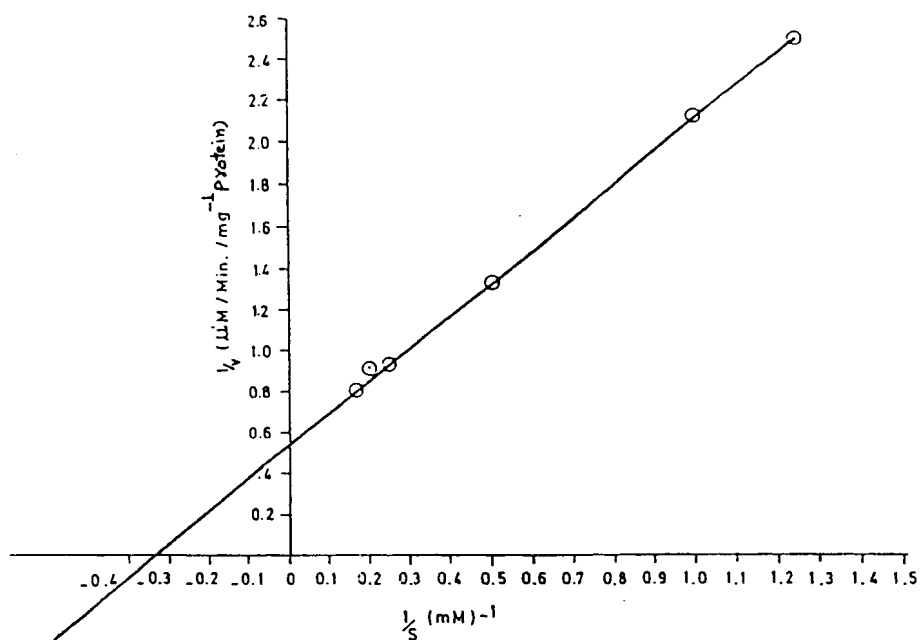


Fig. 4B. Lineweaver-Burk plot for the hydrolysis of p-nitrophenyl phosphate by *W. aegyptia* alkaline phosphatase at: (B) the optimal PNPP concentration range (0.8-5mM), for the pseudo first-order reaction with respect to [PNPP].

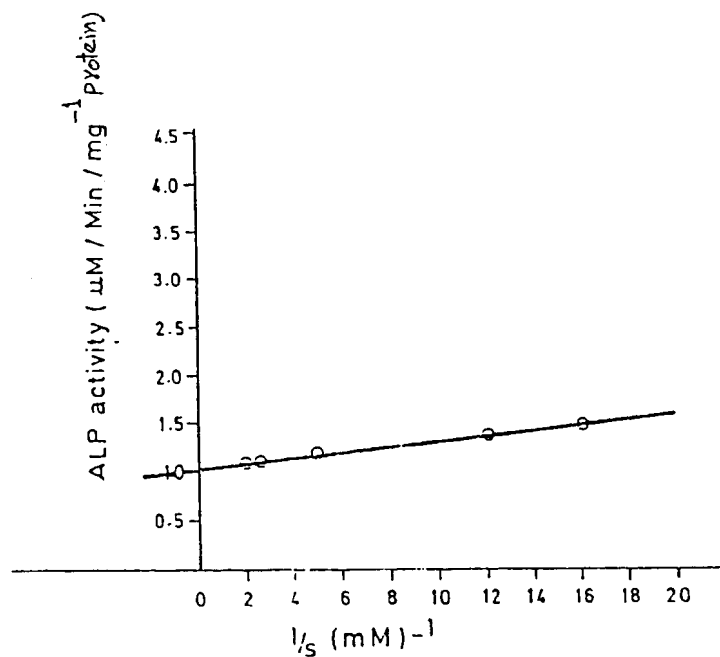


Fig. 4C. Lineweaver-Burk plot for the hydrolysis of p-nitrophenyl phosphate by *W. aegyptia* alkaline phosphatase at: (C) The higher [PNPP] concentration range (8.0-50mM) for the zero order reaction with respect to PNPP concentration.

K_m in case of Fig. 4C. The values for the various kinetic constants for *W. aegyptia* ALP at different rates of reactions are given in Table 2. The K_{cat} catalytic efficiency and (K_{cat}/K_m) values for calf intestinal ALP are significantly lower than the values mentioned in Table 2 (first order reaction) indicating that *W. aegyptia* venom ALP was two times more active than calf intestinal enzyme (Asgeirsson *et al*; 1995). This study showed that the snake venom of *W. aegyptia* is a rich and convenient source of soluble form of alkaline phosphatase.

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خصائص نشاط : أنزيم الكلاين فوسفاتيز في سم الصل الأسود (كوبرا الصحراء) والظروف المثلى لعمل الأنزيم

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المملكة العربية السعودية

ملخص البحث : دُرس أنزيم الكلاين فوسفاتيز (ALP) الموجود في سم ثعبان الصل الأسود وصنف بناءً على مواصفات نشاط الانزيم . وقد لوحظ أن مقدار ٥٠ ميكرو جرام من بروتين السم الخام تكون مناسبة لاعطاء فعالية ذات علاقة بيانية مستقيمة عند درجة حرارة ٢٥م كما تم تحديد الاس الهيدروجيني الأمثل لفعالية الأنزيم ومقداره (pH9.8) أما القوه المثلى للمحلول المنظم المكون من مادة داي ايثانول أمين فكانت (0.9M) وكانت درجة الحرارة المثلى لعمل الأنزيم ٤٠م وكذلك وجد أن حرارة التنشيط تساوي 5663 cal/mol وطاقة التنشيط تساوي 6263cal/mol وكذلك وجد أن التركيز الأمثل للمادة التي يعمل عليها الأنزيم تساوي 8mM ولكن فعالية الأنزيم انخفضت عند تركيزات أعلا من المادة التي يعمل عليها الأنزيم .

وقد قيست في هذه الدراسة معدلات تركيز مادة بيتانيترو فينيل فوسفات (PNPP) لأنظمة مختلفة من التفاعلات أما عوامل نشاط الأنزيم المختلفة (kcat, Vmax, km) ونسبة (kcat/km) فكانت ثابتة عند كل نظام تفاعل.