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AN AMINOPEPTIDASE FROM *AGAVE AMERICANA*, THERMODYNAMIC STUDIES

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Key Word Index—*Agave americana*; Amaryllidaceae; proteolytic enzyme; aminopeptidase; thermodynamic properties.

Abstract—Purified *Agave* aminopeptidase was characterized with respect to the thermodynamic properties of the reaction catalysed by the enzyme. Kinetic studies were conducted at different temperatures ranging from 7.8 to 45°. The energy of activation, E_a, as well as the constants ΔH, ΔF and ΔS were calculated for both the formation of the enzyme–substrate complex, and the dissociation of the enzyme–product complex. Kinetic studies in buffers with varying dielectric constants enabled the determination of the electrostatic as well as the non-electrostatic components of ΔS. These results fit well into the overall kinetic picture of this enzyme-catalysed reaction as reported previously.

INTRODUCTION

The aminopeptidases are, as a group, somewhat more complicated and more difficult to obtain than other proteolytic enzymes. In previous papers [1, 2], this whole situation was discussed to some extent. In addition to the results reported elsewhere [2], the chemical properties of the aminopeptidase isolated from *Agave americana* L. Am. trop., were investigated further and results are reported in this paper. Studies of this kind would serve to elucidate the more specific properties of aminopeptidases and could lead to a better understanding of this group of enzymes.

RESULTS

Kinetic studies were conducted with the aminopeptidase at a variety of temperatures. Results of these studies are presented in Figs 1 and 2 as well as Table 1. According to the results presented in Table 1 it is evident that the apparent Michaelis constant of the enzyme for the substrate remained constant with an increase in temperature. The reaction rate, however, increased with temperature over the temperature range as tested.

By making use of the Arrhenius-plots as indicated in Fig. 2 the energy of activation, E_a, was calculated. For formation of the enzyme substrate complex two values were obtained, a value of 3.84 kcal above 30° and a value of 14.7 kcal below 30°. The corresponding values for the breakdown of the complex, formation of products, E_{a2}, where 3.65 kcal above 30° and 15.12 kcal below 30°. By making use of these values the other thermodynamic

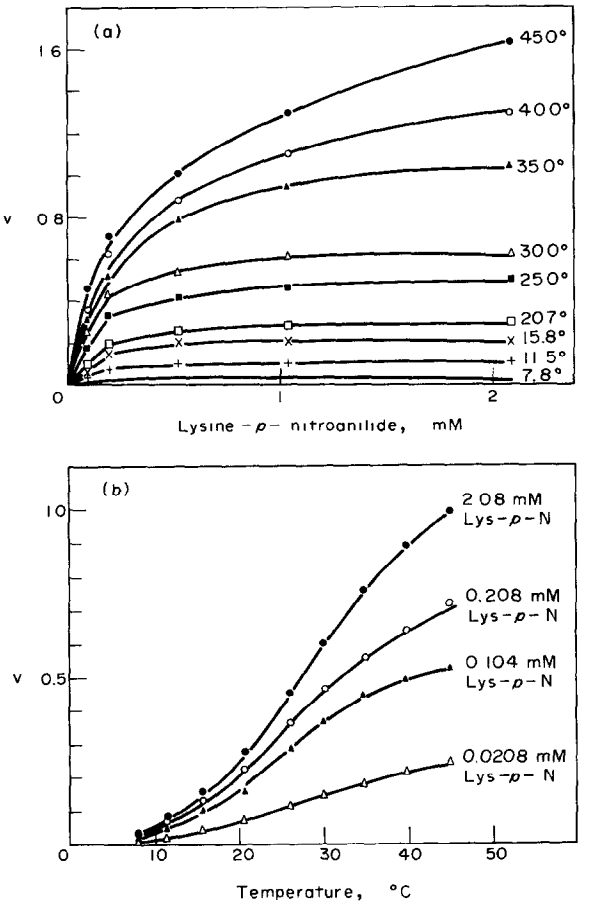

Fig 1. Studies with *Agave* aminopeptidase at different temperatures. The effect of different temperatures on the activity of the enzyme at different substrate concentrations. V = aminopeptidase activity, μmol lysine-*p*-nitroanilide hydrolysed/min/mg protein.

* Part 3 of a series of papers describing the work reported in the PhD thesis submitted to the Rand Afrikaans University by the senior author. For part 2 see du Toit, P. J. and Schabort, J. C. (1978) *Phytochemistry* **17**, 371.

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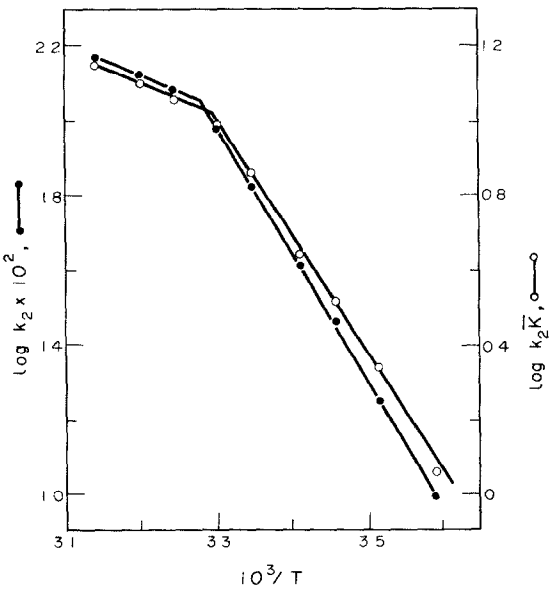

Fig. 2. Studies with *Agave* aminopeptidase at different temperatures. Arrhenius plots for the enzyme. Data from Table 1.

constants of the reaction catalysed were then calculated at 25° and two sets of values, one for the formation of the enzyme substrate complex and the other for the breakdown of the enzyme substrate complex, were obtained. These results are represented in Table 2.

$A\,Q_{10}$ value was also calculated for the enzyme over the range from 20 to 30° and a value of 2.58 was obtained.

The kinetic studies in the presence of varying concentrations of methanol for variation of the dielectric constant of the buffer, were conducted at 25°. Results of these studies are depicted in Figs 3 and 4 as well as Table 3. From these results the effect of variation of the dielectric constant on the kinetic constants of the enzyme can be seen. These results were further evaluated and Fig. 5 represents the variation of $k_2\bar{K}$ as plotted against variation of the reciprocal of the dielectric constant $1/D$. By obtaining the slopes of these lines, it was then possible to evaluate A and A^*[3], and again from the values of these constants, the values of ΔS^*_{es} and (ΔS^*_2) *es*. The values thus obtained were $A = 126800$; $A^* = 44900$; $\Delta S^*_{es} = 14$ entropy units and (ΔS^*_2) *es* = 5 entropy units.

The results of the last mentioned studies, combined with the results of the temperature studies then gave the following pattern.

Table 1. Studies with *Agave* aminopeptidase at different temperatures. Kinetic constants as determined at different temperatures

Temperature	$10^3/T$	K_m ($\times 10^3$ M)	V (μmol/min/mg protein)	k_2 (10^3/sec)	$\log k_2 \times 10^2$	$\text{Log } k_2\bar{K}$
45	3 143	0.104	1 04	1 472	2 168	1 151
39 8	3 2	0 1	0 940	1 33	2 124	1 124
35	3.245	0 108	0 855	1 21	2 083	1 049
30	3 3	0 098	0 667	0 945	1 976	0 984
25 8	2 345	0 092	0 472	0 669	1 926	0 862
20 7	3 41	0 094	0 29	0 41	1 613	0 640
15 8	3 46	0 095	0 215	0 304	1 483	0 506
11 5	3 516	0 085	0 124	0 176	1 246	0 316
7.8	3 56	0 086	0 068	0 097	0 988	0 052

Table 2. Thermodynamic constants of reaction catalysed by *Agave* aminopeptidase as calculated from results of studies at different temperatures

Constant	Value	
	Formation of complex	Breakdown of complex
Energy of activation (E_a and E_{a2})	3.84 kcal (above 30°) 14.7 kcal (below 30°)	3.65 kcal (above 30°) 15.12 kcal (below 30°)
ΔH^* and ΔH^*_2 at 25°	14.11 kcal	14.53 kcal
ΔF^* and ΔF^*_2 at 25°	16.22 kcal	21.65 kcal
ΔS^* and ΔS^*_2 at 25°	−7 *eu*	−24 *eu*

Formation of enzyme–substrate complex:

$$\Delta S^* = 7 \qquad \Delta S^*_{es} = 14 \qquad \Delta S^*_{nes} = 21$$

Dissociation of enzyme–product(s) complex:

$$\Delta S^*_2 = -24 \qquad (\Delta S^*_2)es = 5 \qquad (\Delta S^*_2)nes = -29$$

DISCUSSION

The calculated thermodynamic constants (Table 3) agree well with those of other hydrolytic enzymes with respect to the order of magnitude [4]. All the thermodynamic constants were of course only obtained for the forward reaction because it is not possible to obtain these values for a backward reaction in the case of these hydrolytic enzymes. In literature, no comparable studies could be found that were specifically conducted with aminopeptidase enzymes. The problem thus arises that these obtained values cannot be compared with values of a similar enzyme.

The discontinuity in the Arrhenius plots can be explained by a few different postulates as contained in the explanations of Dixon and Webb [4]. A similar discontinuity was observed by Massey [5] during studies with fumarate hydratase. This type of discontinuity can be explained by postulating a phase change in the solvent which is not possible in this case; otherwise it might be caused if there are two parallel reactions taking place at the same time, by virtue of the existence of two separate active centres catalyzing the two reactions. The temperature coefficient of these reactions must differ to be able to explain the sudden discontinuity observed. In this latter case the higher activation energy should be observed at the higher temperature which is not the case as can be seen from Table 3. The only explanations that do fit the reported data, is either to postulate that the reaction system consists of two sequential reactions with different temperature coefficients, or that two different forms of enzyme

Table 3. Studies with *Agave* aminopeptidase at different methanol concentrations. Kinetic constants as determined at these different concentrations.

% Methanol (v/v)	K_m ($\times 10^3$ M)	V (μmol/min/mg protein)	k_2 (10^3/sec)	$\log k_2 \times 10^2$	$\log k_2\bar{K}$
0	0 1	0 582	0 825	0 916	0 916
1	0 1	0 625	0 885	0 947	0 947
5	0 1	0 617	0 875	0 942	0 942
10	0 103	0 614	0 87	0 94	0 927
15	0 104	0 588	0 833	0 921	0 904
20	0 116	0 544	0 77	0 887	0 882
25	0 135	0 51	0 722	0 859	0 728
30	0 179	0 44	0 622	0 831	0 648

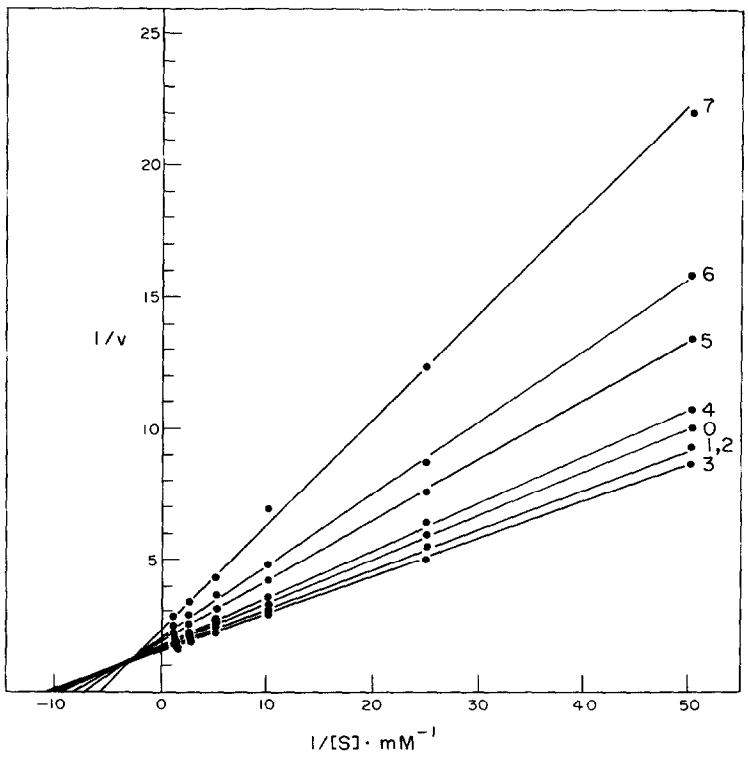

Fig. 3. Studies with *Agave* aminopeptidase at different methanol concentrations. Determination of the kinetic constants using the Lineweaver–Burk procedure. 0: none, 1: 1%, 2: 5%, 3: 10%, 4: 15%, 5: 20%, 6: 25% and 7: 30% methanol (v/v).

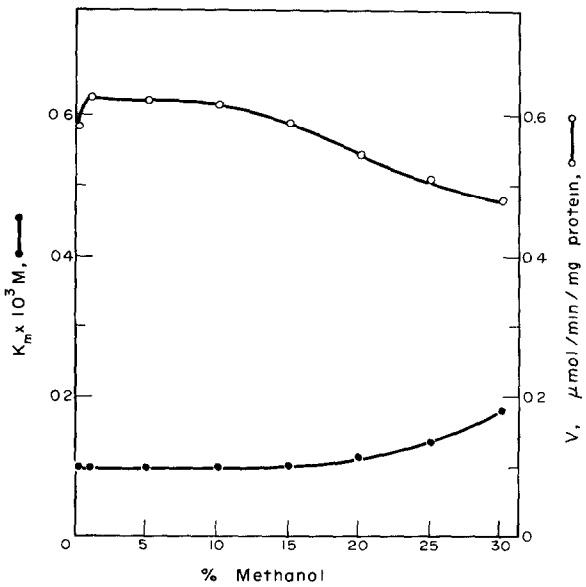

Fig. 4. Studies with *Agave* aminopeptidase at different methanol concentrations. The variation of K_m and V with an increase in methanol concentrations.

can take part in the catalytic process, which implies that there is a structural change of protein structure caused by an increase in temperature. A point supporting the first of these last hypotheses is that one would expect the reaction with the higher temperature coefficient to be faster at the higher temperatures with the result that the overall reaction rate will then be dictated by the other reaction, causing this last reaction's activation energy to be observed at these higher temperatures. Such a system can also be explained theoretically [6]. There seem to be a few factors arguing against a structural or conformational change of the enzyme itself to cause the observed discontinuity. For instance the relative Michaelis constant of the enzyme for the substrate could be expected to change concomitantly with the structural change. This is however not conclusive and this whole picture will only be clarified if further studies relating to the conformation of the enzyme were carried out over this temperature range as studied.

These observations, relating to the non-linear increase in reaction velocity with increase in temperature, may also explain the somewhat high Q_{10}-value that was observed for the enzyme-catalysed reaction.

An interesting observation during these studies at different methanol concentrations was that reaction rate of the enzyme-catalysed reaction increased over the range from 0–10% methanol and that the apparent affinity constant of the enzyme for its substrate changed very little. A decrease in the value of the dielectric constant

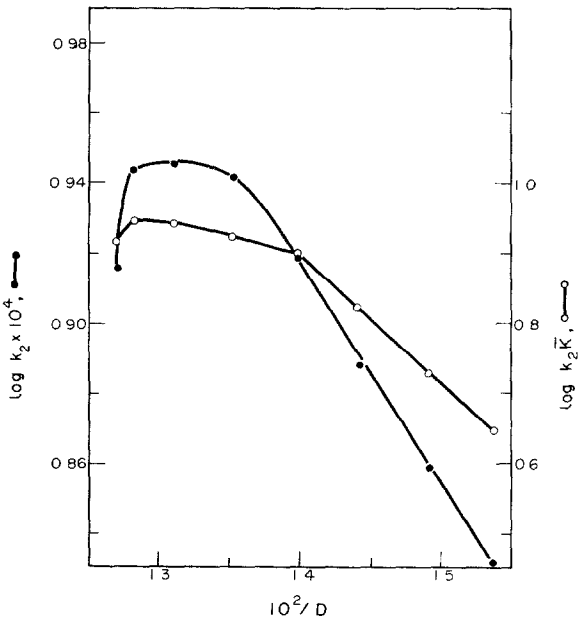

Fig. 5. Effect of decreasing the dielectric constant of buffers on the catalytic activity of *Agave* aminopeptidase. D = dielectric constant, k_2 = velocity constant $\bar{K}$ = the reciprocal of the K_m-value, i.e. an affinity constant.

thus caused an increase in reaction rate up to a certain limiting value.

These observations were made several times during different experiments. The increase in activity was however, at most only 6%, and an increase in methanol concentration above 10% led to the expected decrease in reaction rate as well as a change in the other kinetic parameters. The logarithm of the velocity constant, k_2, decreased linearly as the value of $1/D$ was increased. If the obtained entropy values are taken into account, a few interesting deductions can be made. The positive value for the ΔS^*_{es}-value, 14, suggests a neutralization of charge during complex formation, a most applicable deduction if one thinks about the amino terminal structure of a peptide. Previous results also suggested the participation of a histidine group in the catalytic process of the enzyme [2]. The value of 5 for (ΔS^*_2) *es* suggest a separation of charges during dissociation of the reaction complex and in this case, similar charges. The situation is also possible considering the nature of the groups formed during the reaction catalysed by this enzyme. The values for the non-electrostatic terms are as could be expected for a bimolecular reaction and it does not need any postulation of drastic structural changes during the reaction. However, the possibility of some structural changes occurring is not excluded.

The above-mentioned effects, considering the electrostatic terms, also gives an explanation for the previously reported ionic strength effects [2]. The enzyme was observed to display maximal catalytic activity at concentrations of 0.1 to 0.2 M and even higher concentrations of buffers.

These observed thermodynamic properties of the aminopeptidase fit well into the overall pattern regarding the general kinetic picture as observed for the enzyme.

EXPERIMENTAL

Agave aminopeptidase was isolated from *Agave americana* and the purity of the enzyme was determined by electrophoresis, gel filtration and ultracentrifugal analysis [1]. The chemical properties of the enzyme with respect to substrate specificity and some factors governing the mechanism of action were also reported [2]. Protein concn as well as enzyme activity determinations were carried out as described in ref [1, 2]. The effect of temp. (7, 8 to 45°) on the activity of the aminopeptidase was tested, using lysine-*p*-nitroanilide as substrate. The buffer used during these studies was Tris–maleate [7], 0.18 M, pH 7.2. Results were analysed according to the procedures as described in refs [3, 4, 8, 9]. To determine the effect of variation of the dielectric constant on the activity of the *Agave* aminopeptidase, MeOH was used [10]. Preliminary studies were conducted at 30° with 2 μM lysine-*p*-nitroanilide as substrate in 0.08 M Tris-buffer, pH 7 5 Kinetic studies were conducted at 25° with lysine-*p*-nitroanilide as substrate in 0.18 M Tris–maleate buffer, pH 7.2. During these studies 8.1 μg purified aminopeptidase was used per assay and the substrate concn was varied from 0.02 mM to 1 mM.

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