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Inactivation of α-amylase from *Bacillus amyloliquefaciens* at low moisture contents

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ABSTRACT

The thermal inactivation characteristics of dehydrated α-amylase in solid state at various moisture contents was studied at temperatures between 135 and 150°C. The inactivation kinetics show a first order decay. The enzyme was found to be more thermostable than in aqueous solution. The z-value dependency with moisture content has shown some fluctuactions, having an absolut minimum of 19°C at 23.9% moisture.

1. INTRODUCTION

α-amylases [(1-->4) glucan 4-glucanohydrolases, EC 3.2.1.1.] catalyze the breaking of α-1,4-glucosidic bonds in polisaccharides

molecules, except the one located at the extreme of the molecules, in a more or less random manner [1]. This type of enzymes are widely used in the sugar, textile and brewery industries. Due to these industrial applications, thermal stability of these enzymes is well reported in literature [2-7].

However, the effect of moisture content on heat resistance of α-amylase in solid phase is not reported in literature. Examples of the effect of water removal from proteins on their thermal stability includes: porcine pancratic lipase [8], Phospholipase D [9], pancreatic and wheat grain ribonuclease [10], ß-lactoglobulin [11], mioglobin [12] and α-amylase from *Bacillus lichneniformis* [13]. In all these cases dehydration of the proteins caused their thermal stabilization.

In this work we screened the thermal inactivation characteristics of dehydrated α-amylase with a view to a possible development of a Time-Temperature Integrator (TTI) for use at temperatures between 100 to 140°C, based on this enzyme. For such a system to work as a TTI, it is necessary to have z-value of 10°C and an adequate D-value in the temperature range of interest [14]. For more details about the use of this type of systems, see the work of De cordt *et al.* [15]. This means, in our particular case that the enzyme must be very thermo-stable, since it must operate at high temperatures. One way to stabilize one enzyme is by drying it, which is why we are trying to use such a system. Other work is being done with other strategies such as enzyme stabilization by immobilization and/or the presence of the substrate or substrate analogues [15]. A sucessfull TTI operating at pasteurization temperatures was already developed [16], based on immobilized horseradish peroxidase.

2. MATERIALS AND METHODS

The α-amylase from *B. amyloliquefaciens* (EC 3.2.1.1.) used in this work was produced by Sigma (St. Louis, Mo 63158, USA). 0.25 ml of a 0.13 mg/ml water solution of amylase in crimp top vials from chrompack was used for lyophilization. The lyophilized amylase was

equilibrated at 4°C above the following standard salt solutions : lithium chloride, potassium acetate, potassium carbonate, sodium chloride ammonium sulphate and potassium chloride, of water activity (a_w) 0.11, 0.23, 0.43, 076, 0.82 and 0.88 [17], respectively, for six days. The inactivation experiments were performed on the seventh day.

For each a_w, prior to the inactivation experiments the vials were tightly closed, as fast as possible, to avoid atmosphere changes, using chrompack aluminium caps and hand crimper. The closed vials were transferred directly from 4°C to an oil bath, at the required temperature for the particular experiment. At predetermined time intervals the vials were quickly transferred to an ice bath. After cooling, the vials were opened, Tris-HCl buffer (pH 7.2) added to each one and kept in the ice bath until analysis of remaining activity. This experiment was duplicated at each temperature for each a_w.

The activity of the amylase was measured according to the procedures nº 576 or nº 577 of Sigma Diagnostics. The initial reaction rate (Δ OD/min) at 30°C and at 405 nm was used to express the enzyme activity.

For determination of the sorption isotherm the enzyme was dried above P_2O_5 at 4°C during two weeks, after which 100 mg, in duplicate, were equilibrated at the same temperature above standard salt solutions of a_w 0.11, 0.23, 0.34, 0.43, 0.59, 0.64, 0.76, 0.82, 0.88 and 0.92 [17]. The moisture content was determined gravimetrically, weighing the samples every day, starting on the fourth day of equilibration. Before weighing, the closed recipients containing the enzyme, were equilibrated to room temperature in a desicator during 30 min. The final moisture content, for each a_w, was determined as the average of the last two constant weights for each duplicate.

The amylase inactivation kinetics was analyzed considering a first order reaction rate:

$$A/A_o = \exp(-k^*t) \qquad (1)$$

where :A=Total residual activity of the enzyme (Δ OD/min),
A_o=Total initial activity of the enzyme (Δ OD/min),
k=Reaction rate constant (min^{-1}),

t=Time (min).

For comparasion with literature results, the conventional two-step method was used for determining the z-value. Using the linear regression procedure of the SAS Software package [18], the reaction rate constant for each temperature was estimated from the corresponding experimental data. The D-values were calculated according to the definition:

$$k = Ln(10)/D \quad (2)$$

where : D=Decimal reduction time (min).

The temperature dependence of the D-value is exponential with z-value and therefore:

$$D = D_{ref} * 10^{(T-Tref)/z} \quad (3)$$

where: D=Decimal reduction time at temperature T (min),
D_{ref}=Decimal reduction time at a reference temperature (min),
T=Temperature (°C),
Tref=reference temperature (°C),
z=Temperature increase needed to reduce the D-value by one log-unit.

3. RESULTS AND DISCUSSION

Linearity between enzyme concentration and initial activity and the behaviour of amylase during lyophilization and stability above standard salt solutions was tested and the best conditions were chosen.

Plots of logarithm of residual activity *versus* heating time showed a linear behaviour for all temperatures and water activities, validating the assumption of first order reaction rate. The initial value

fell outside the strait line suggested by all points and therefore was not considered for the linear regressions. An example is shown in Fig. 1 for a_W=0.76. In table 1 are shown the kinetic parameters obtained for all the cases studied. The temperature range of inactivation (135-150°C) is higher when compared with inactivation in aqueous solution (the enzyme rapidly inactivates at 90°C [7]. Even for more heat-resistant α-amylases from thermophilic organisms, such as *B. licheniformis*, the enzyme is not stable in solution in the temperature range used in this work [2,15]. For soluble and covalently immobilized α-amylase, some authors reported a biphasic inactivation profile [3,6-7]. In earlier work, we also reported a biphasic inactivation for peroxidase in similar conditions to that used now for α-amylase [19]. Although the inactivation temperatures are practically the same in this case as with peroxidase, the D-values are always lower for α-amylase, indicating that this enzyme is clearly less stable in dried state. The same conclusion can be taken when comparing thermal inactivation of both enzymes in solution.

In order to evaluate the amount of water bound to the protein and its effect on thermal stability, the sorption isotherm of the enzyme

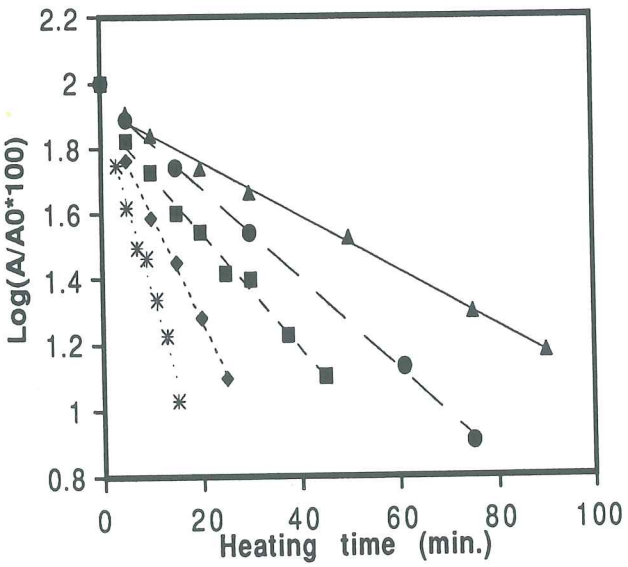

Fig. 1. Heat inactivation of α-amylase at moisture content of 16% at 135°C (▲), 140°C (●), 145°C (■), 150°C (◆) and 155°C (✳).

Table 1. Kinetic parameters obtained for inactivation of α–amylase from *Bacillus amyloliquefciens* and their individual 95% confidence interval.

Aw	Temp. (°C)	-Intercept	K (min^{-1})	D (min.)	z (°C)	r#
0.11	135.0	4.40±0.082	0.020±0.0020	114.80	28.97	0.997
	140.0	4.29±0.140	0.032±0.0033	73.01		
	145.0	4.24±0.195	0.048±0.0100	47.90		
	150.0	4.34±0.103	0.065±0.0068	35.12		
0.23	135.0	4.44±0.051	0.016±0.0011	147.75	22.54	0.988
	140.0	4.36±0.156	0.033±0.0033	70.76		
	145.0	4.20±0.160	0.044±0.0070	51.82		
	150.0	4.43±0.232	0.077±0.0104	29.87		
0.43	135.0	4.44±0.078	0.020±0.0017	117.01	28.41	0.999
	140.0	4.37±0.052	0.031±0.0017	69.88		
	150.0	4.44±0.181	0.067±0.0090	34.30		
0.76	135.0	4.44±0.056	0.019±0.0012	119.55	24.46	0.992
	140.0	4.50±0.078	0.032±0.0019	72.92		
	145.0	4.35±0.086	0.040±0.0033	57.06		
	150.0	4.43±0.091	0.075±0.0054	28.69		
	155.0	4.40±0.141	0.127±0.0143	18.11		
0.82	140.0	4.44±0.056	0.036±0.0035	63.60	30.79	0.986
	145.0	4.47±0.133	0.059±0.0053	39.23		
	150.0	4.46±0.106	0.076±0.0073	30.11		
0.88	135.0	4.37±0.064	0.017±0.0011	137.17	18.71	0.981
	140.0	4.48±0.100	0.031±0.0031	73.65		
	145.0	4.60±0.149	0.044±0.0056	52.58		
	150.0	4.72±0.310	0.117±0.0206	19.75		

#r stands for correlation coefficient.

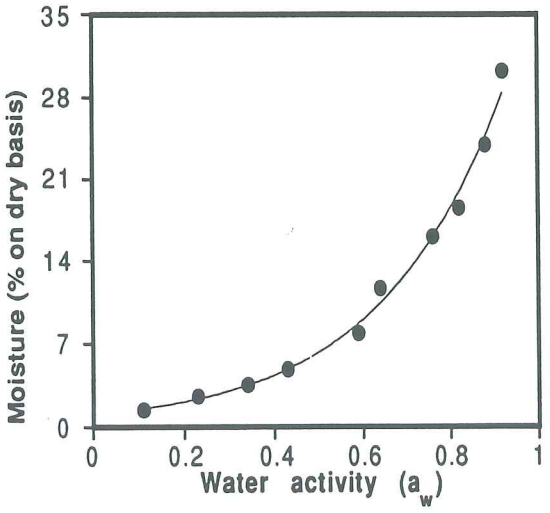

Fig. 2. Sorption isotherm of α-amylase at 4°C.

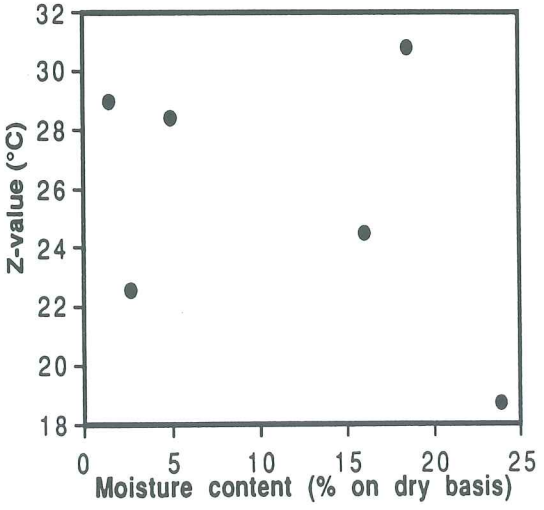

Fig. 3. z-value for α-amylase as a function of moisture content.

was determined and is presented in Fig. 2. As can be seen in Fig. 3 no apparent relation can be obtained between the z-value and moisture content with these data. It is however evident that a minimum value exists at the highest moisture content studied. The activation energy for *B. licheniformis* α-amylase inactivation, during drying using malto-dextrins as support material, was shown to be an exponential increasing function with moisture [13]. However, the moisture content range considered was different from our case, reason why no comparisons can be made. The z-value for peroxidase was found to have a maximum at a_w=0.76 (moisture content of 16.2%) [19]. Results similar to that obtained for peroxidase were reported for wheat grain ribonuclease [10].

Although, water removal from proteins stabilizes them against heat, the reason why this happens is still unknown but maybe due to the influence of water on the three dimensional structure and its possible participation in the reactions leading to irreversible thermal inactivation.

4. CONCLUSION

Dehydrated α-amylase from *Bacillus amyloliquefaciens* is very thermostable at low moisture contents, compared to inactivation in aqueous solution. However this enzyme is less stable than horseradish peroxidase at the same conditions. The z-value as a function of moisture content has a different profile for both enzymes. The variation range of the z-value in the whole range of water activity (moisture content) is also larger with peroxidase. Correlation between the z-value and moisture content or water activity is not evident for the data. This requires further statistical analysis to interpretate correctly the apparent random variation found. It is known that z and D-values are highly colinear and therefore the two-step linear regression can be responsible for apparent random variations.

5. REFERENCES

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