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Thermal Inactivation of Lectins (PHA) Isolated from *Phaseolus vulgaris*

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ABSTRACT

The influence of the thermal process on the loss of ability to bind a carbohydrate target was studied on lectins (PHA) purified from Phaseolus vulgaris *seeds. Thermal inactivation of aqueous solutions of pure PHA occurred according to a biphasic first-order mechanism, the thermodynamic parameters, at pH 7·3, being as follows:* $\Delta H_1^* = \Delta H_2^* = 86{\cdot}2\,kcal\,mole^{-1}$, $\Delta S_1^* = -54{\cdot}04\,cal\,deg^{-1}$ *and* $\Delta S_2^* = -56{\cdot}71\,cal\,deg^{-1}$. *The first-order rate constants appeared to be dependent on pH (minimal around 7) and divalent cations. All different subunits constituting the whole PHA were inactivated at the same rate. The biphasic nature of this process is independent of the presence of 10 mM* Ca^{++} *or* Mg^{++} *and appeared to indicate a discrete aggregation of PHA molecules.*

INTRODUCTION

Raw, or poorly cooked, leguminous seeds are toxic when consumed by humans and other mammals (Kakade & Evans, 1966; Noah *et al.*, 1980; Aletor & Fetuga, 1984). Several antinutritional factors may be present, among which lectins appear to be the most deleterious, especially in beans (Pusztai & Palmer, 1977; Jaffé 1979; Pusztai *et al.*, 1979; Weiser, 1984). Consumption of legumes is historically established and the cooking times needed to make beans edible were, indeed, determined empirically

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centuries ago. Nevertheless, industrial cooking processes, especially that of composite meals such as weaning foods, necessitate an accurate knowledge of heat inactivation of lectins. Few assays have been performed on the lectins *in situ* (Antunes & Sgarbieri, 1980; Grant *et al.*, 1982) but it seemed to us relevant to study a more simple model.

The aim of the work described in this paper was to determine the pattern of loss of carbohydrate binding ability in the course of thermal processing of lectins purified from *Phaseolus vulgaris*.

MATERIAL AND METHODS

Preparation of PHA

Kidney beans (*Phaseolus vulgaris*), cultivar Lignor blanc, were purchased from a single source (La Lyre, Ste Cécile, France). Fetuin was purified from bovine fetal sera according to the two-step procedure of Marti *et al.*, (1973) and coupled to Sepharose 4B–Cl (Pharmacia) activated by cyanogen bromide (March *et al.*, 1974). PHA was prepared from a saline extract of beans by affinity chromatography on this support (Pusztai & Stewart, 1978). The lectin was eluted by lowering the pH to 3·0, dialyzing exhaustively, then freeze-drying. Its purity was checked on SDS–PAGE and by spectrophotometry, assuming $A^{1\%}_{280nm} = 11{\cdot}4$ (Leavitt *et al.*, 1977).

Buffers

The ionic strength of all buffers used in thermal processing was 0·1. Buffer compositions were calculated according to the temperature dependence of respective pK_a, as published elsewhere (Gueffroy, 1978). Apparent pH values were checked experimentally at 81 °C.

Heat inactivation kinetic measurements

Ten millilitres of a PHA solution at a concentration of 1 mg/ml, at a definite pH within the range 4·0–9·3, were incubated in a water bath, which was thermostatted at the indicated temperature ±0·1 °C. At different times, 1 ml aliquots were immediately frozen in liquid nitrogen, then stored at −80 °C until assay. After thawing, each aliquot was spun at 10^4 g for 5 min and assayed for residual active lectin; each supernatant was chromatographed through 250 μl of fetuin-Sepharose gel at ambient

temperature (0·5 × 10 cm, Econocolumn, Biorad). After extensive washing, active PHA was eluted by means of a pH 3·0 glycine buffer and determined spectrophotometrically.

Calculations of kinetic rate constants

Logarithms of active PHA concentration were plotted versus heating time. The graphs appeared biphasic. The initial slope (k_i) was considered as the apparent constant of two simultaneous reactions, such as:

$$k_i\,[P_0] = k_1\,[P_1] + k_2\,[P_2] \quad (1)$$

and:

$$[P_0] = [P_1] + [P_2] \quad (2)$$

where:

$[P_0]$ = initial PHA concentration.

$[P_1]$ = part of $[P_0]$ entering the fast reaction.

$[P_2]$ = part of $[P_0]$ entering the slow reaction.

k_2 was calculated from the slope of the slow reaction. $[P_2]$ was determined by extrapolations of the slow reaction to the ordinate axis. k_1 and P_1 were computed from eqns (1) and (2).

Haemagglutination assays

These were performed using trypsinized rabbit erythrocytes (Gordon *et al.*, 1972) treated with glutaraldehyde (Turner & Liener, 1975). Haemagglutinin tests were performed on serial twofold dilutions of lectins solutions for 2 h at ambient temperature. OD^{650nm} values were plotted versus the dilution scale.

Electrophoresis

After chromatography through fetuin-Sepharose, aliquots of residual active PHA, containing 25 μg of lectin, were precipitated by 10% TCA (v/v) and subjected to SDS–PAGE according to Laemmli (1970).

RESULTS AND DISCUSSION

A typical thermal inactivation reaction pattern of the PHA is depicted in Fig. 1. This pattern was obtained at 82 °C and pH 7·3, with an initial

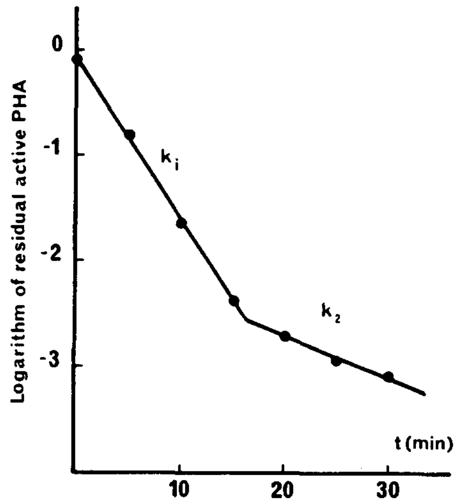


Fig. 1. Semi-logarithmic representation of thermal inactivation of PHA at 82 °C and pH 7·3.

PHA concentration of 1·10 mg ml^{-1}. It might be interpreted by two concomitant first-order reactions with different rate constants. This experiment was started again at several temperatures, ranging from 74 °C to 86 °C at the same pH, 7·3. The Arrhenius plot (Fig. 2) allowed calculation of the thermodynamic parameters of this inactivation, which were $\Delta H_1 = \Delta H_2 = +86{\cdot}2$ kcal mole^{-1}, $\Delta S_1 = -54{\cdot}04$ cal deg^{-1} and

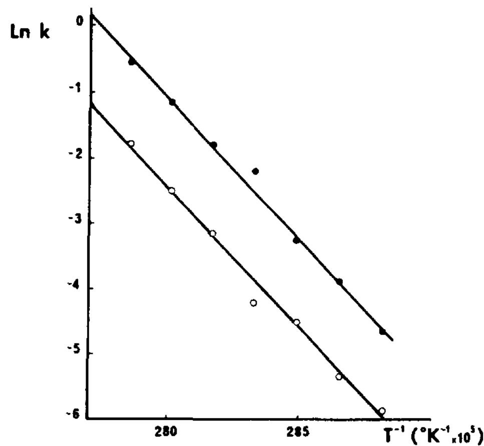


Fig. 2. Arrhenius plots of thermal inactivation at pH 7·3.

TABLE 1
Evolution of Residual Active PHA Concentration (mg ml^{-1}) Kept at Ambient Temperature After Thermal Processing at 82 °C and pH 7·2. Indicated Times Started from the End of Heat Treatment

Initial concentration (before heating)	*Residual concentrations*				
	0 h	*0·5 h*	*2 h*	*5 h*	*24 h*
0·982 Mg ml^{-1}	0·176	0·174	0·175	0·176	0·157

$\Delta S_2 = -56{\cdot}71$ cal deg^{-1}. As assessed from Table 1, this inactivation was not reversible within 24 h at pH 8·0 (the initial pH value was 7·3 at 82 °C) and ambient temperature.

The influence of pH was studied within the range 4·0–9·3 (Fig. 3). The rate constants were too high to be measurable at pH 4·0, declined at higher pH to a minimum (about pH 7·3) and then rose again. It must be

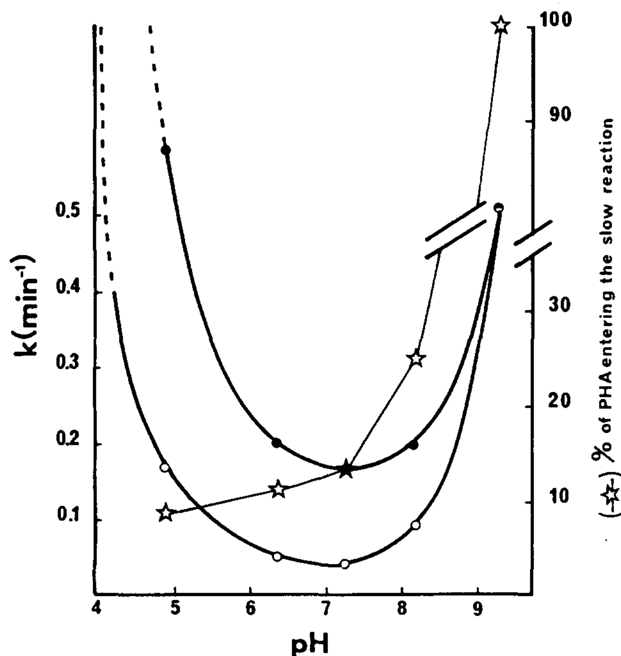


Fig. 3. Influence of pH on rate constants of thermal inactivation of PHA at 82 °C (●, k_1; ○, k_2). Part of the PHA molecules enter the slow reaction (for details, see text: 'Material and Methods' section).

pointed out that k_1 and k_2 became closer as the pH rose in such a way that, above pH 9·0, the kinetics of thermal inactivation of PHA appeared quite linear. Concomitantly, the part of the PHA molecules entering the slow reaction increased with the PH.

The biphasic shape of the kinetics might be explained by several hypotheses.

The first hypothesis was supported by our knowledge of the PHA structure: lectins from *Phaseolus* are in tetrameric form. Each tetramer is a statistical assembly of two types of protomer differing in their molecular weights and properties. One is specific for erythroagglutinating properties (E), the other is responsible for mitogenic properties towards lymphocytes (L). Thus, pure PHA is, in fact, a mixture of five kinds of tetramer (L_4, L_3E, L_2E_2, LE_3 and E_4) (Leavitt *et al.*, 1977). Accordingly, one might suppose that all the different tetramers were not inactivated at the same rate. But, as shown by electrophoresis (Fig. 4), no change in protomer ratios within the residual active PHA could be observed during the course of the thermal process at 82°C. Furthermore, fetuin affinity and haemagglutination titres were lost simultaneously (Fig. 5). The biochemical heterogeneity of PHA could not account for the biphasic graph of thermal inactivation.

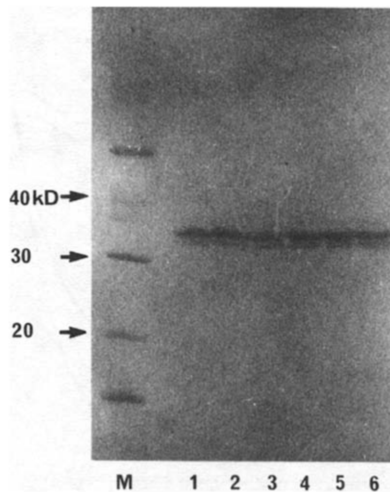


Fig. 4. SDS—polyacrylamide electrophoresis (15% acrylamide) of residual active PHA in course of heating process at 82°C and pH 7·3. Lines 1 to 6 correspond, respectively, to 0, 5, 10, 15, 20 and 30 min heating times. Molecular weights of markers (M) are indicated by the arrows.

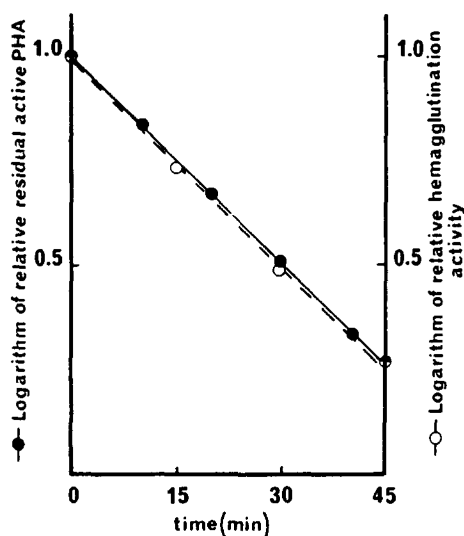


Fig. 5. Comparison of losses of fetuin affinity and haemagglutination titres in course of heating of PHA at 78 °C. The heating was stopped before the fast reaction was achieved.

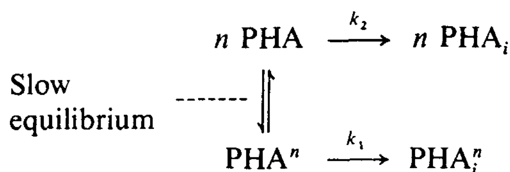
Another hypothesis concerned the divalent cations associated with PHA. Contrary to earlier work devoted to the concanavalin A structure (Shoham *et al.*, 1979), the metal content of the active PHA, purified from *Phaseolus vulgaris* (Assouad *et al.* (1985), as well as that of lectins from other beans (Jaffe *et al.*, 1977) and even concanavalin A (Brewer *et al.*, 1983), may be quite variable. Two aliquots of PHA solutions were subjected to 24 h preincubations, respectively, with 10 mM Mg^{++} and 10 mM Ca^{++}, then brought to 82 °C and pH 7·3. When compared with control (preincubated without divalent cations), the first-order rate constants were lowered by Ca^{++} and chiefly by Mg^{++}. Furthermore, the proportion of PHA molecules entering the slow reaction was increased (Table 2). The kinetic processes remained biphasic in the presence of 10 mM divalent cations so this second hypothesis appears inadequate.

The last hypothesis rests on the existence of an equilibrium between two conformational states of PHA molecules. From the results listed above, it may be supposed that the interconversion rates are fairly slow and weakly dependent on temperature, but highly dependent on pH and divalent cations. Furthermore, the rate constants for the two observed reactions of thermal inactivation differed only in their entropy increments. They may therefore involve identical reactant molecules differing only in their state

TABLE 2
Influence of 10 mM Mg^{++} or Ca^{++} on the Thermal Inactivation of PHA at pH 7·3 and 82°C.

	k_1 (*min*$^{-1}$)	k_2 (*min*$^{-1}$)	*Per cent of initial PHA entering the slow reaction*
Control	0·171	0·043	16·3%
10 mM Ca^{++}	0·123	0·037	26·0%
10 mM Mg^{++}	0·088	0·035	46·4%

of order. It has been known for a long time that PHA precipitates slowly in the course of dialysis (Manen & Miege, 1977), i.e. upon removal of divalent cations. This precipitation is also maximal around neutrality and weakens as the pH is raised above 8. Although we could not present experimental evidence, a discrete process of autoassociation of PHA molecules would account for the biphasic reaction of thermal inactivation of PHA according to the following hypothetical scheme:



In conclusion, the thermal inactivation of pure aqueous solutions of PHA is a first-order reaction exhibiting a very high enthalpy increment. The values of the rate constants were higher than that computed from a previous report concerning the effect of heating of whole beans on their haemagglutination activity (Grant *et al.*, 1982). This means that lectins *in situ* are largely protected from heat inactivation. The mechanism of such a protection within the bean now remains to be established.

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