Thermal Inactivation Parameters for cod pepsin, cod pepsinogen and porcine pepsin

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Abstract: - The thermal inactivation parameters of cod pepsin, cod pepsinogen and porcine pepsinis were compared at pH 2, 40-65^{o}C. Cod pepsinogen and cod pepsin were less stable than porcine pepsin mainly as a result of large value of activation entropy.

Key-Words: - pepsin, pepsinogen, inactivation, rate constant, porcine, activation free energy, activation enthalpy, activation entropy, cod

1 Introduction

Proteases are the most extensively used enzymes in industrial application. At present, industrial proteases are mainly derived from microbial, plant and animal sources. During the last two decades, aquatic organisms have been recognised as a new sources of proteases [10,3]. Cold-adapted fish proteases are of interest owing to their greater proteolytic activity towards native protein substrates and a lower activation energy for enzymatic compared with proteases from mammalian or microbial sources [9].

Thermal inactivation is perhaps the most common reason for inactivation. From a biotechnological view point, thermal inactivation is by far the most frequently encountered cause of enzyme inactivation in industry [1]. Knowledge about thermal inactivation is important in order to minimise enzyme inactivation during isolation, application and storage [8]. Such a study is also important for the understanding of the structure-function relationship of enzymes. Most enzyme inactivations occur as first order reactions.

Many studies have been reported on purification and characterization of fish pepsin [5], but only few studies reported its thermal inactivation. This paper reports thermal inactivation parameters for cod pepsin, cod pepsinogen and its comparison with porcine pepsin.

2 Methodology

2.1 Materials and Method

Purification of cod pepsin was carried out on ion exchange of Amberlite-50 as described in Amiza and Owusu Apenten [2]. Inactivation of pepsin activity as a function of time were performed at four different temperatures. Cod pepsin and pepsinogen were studied in the temperature range of 40-55^{o}C, while porcine pepsin was studied in the temperature range of 50-65^{o}C. The concentration of protein was 0.25 mg/ml and 0.8 mg/ml for cod pepsin and cod pepsinogen, respectively (determined by the modified Lowry method [8]). For porcine pepsin, a stock solution of 0.1 mg/ml was prepared. The protein concentrations were chosen in a way that after 5-fold dilution in the buffer, the activity was in the range of 0.4-0.5 U (1 U is defined as the amount of enzyme causing an increase of 1.000 in absorbance at 280 nm per 30 min reaction time for 1-cm pathlength). Enzyme solution (1 ml) was added to a heated buffer solution (4 ml of 50 mM KCl/HCl buffer, pH 2) in a 50-ml stoppered conical flask. Samples (100 μl) were transferred to an

Eppendorf tube at various time intervals and then cooled in an ice-bath prior to the residual activity being assayed. The important data obtained from heat inactivation studies is the residual activity versus time with the activity at 0 time considered as 1.

2.2 Data Analysis

Determining enzyme inactivation involves estimating the rate constant for inactivation (k) for the reaction:

$$N \xrightarrow{k} I \qquad (1)$$

where N is native enzyme and I is inactivated enzyme. Enzyme inactivation may occur in single-step inactivation or consecutive-step. In this study, enzyme inactivation is assumed to occur in a single-step.

2.2.1 Determination of rate constant

For this type of inactivation, the general rate equation with n-th order is:

$$-\frac{d[N]}{dt} = k\ [N]^n \qquad (2)$$

For 1-st order reactions (i.e., n=1), integrating Equation (1) yields:

$$\ln\frac{N}{N_o} = -k\ t + C \qquad (3)$$

(1.9)

where $[N_o]$ and $[N]$ is the activity of native enzyme at time 0 and t respectively, t is time and C is a constant. A plot of ln (residual activity) versus t, gives a slope of -k and a constant C as the intercept. The plot should be linear if the reaction order is really first order.

For 2-nd order reactions, (i.e., n=2), the integration produces:

$$\frac{1}{[N_o]} - \frac{1}{[N]} = -k\ t + C \qquad (4)$$

Thus, a plot of the reciprocal of residual activity versus t, gives a slope of -k and a constant C as the intercept.

Determination of n is done by fitting linear regression to the data using Cricket Graph™ where the value of n is limited to either 1 or 2.

2.2.2 Activated complex theory or Transition state (Eyring) theory

According to transition state theory, an activated complex (or transition state) formed in passing from reactants and product molecules. There is an equilibrium between reactants and activated complex. For enzyme inactivation, the equilibrium can be represented as:

$$N \Leftrightarrow N^{\#} \Rightarrow U \quad \text{or} \quad U \Leftrightarrow U^{\#} \Rightarrow I$$

where $N^{\#}$ and $U^{\#}$ are the transition states when unfolding ($N \Leftrightarrow U$) or $U \Leftrightarrow I$ reaction are rate-limiting. Thermodynamic activation parameters apply for the reversible reaction for the formation of $N^{\#}$ or $U^{\#}$ (i.e. $\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$). The relationship between k and the activation free energy ($\Delta G^{\#}$) at a particular temperature is given by:

$$k = \left(\frac{KT}{h}\right)\exp^{(-\Delta G^{\#}/RT)} \qquad (5)$$

or

$$\Delta G^{\#} = -RT\ln(kh/KT) \qquad (6)$$

where K is Boltzmann constant and h is Planck's constant.

Arrhenius and Eyring equations are similar but they are not identical. The transition state theory or Eyring theory relates the irreversible kinetic stability to a range of thermodynamic activation parameters. Derivation using this theory yields the relation between the difference in enthalpy of activation for inactivation and the Arrhenius activation energy i.e.:

$$E_a^{\#} = \Delta H^{\#} + RT \qquad (7)$$

Fig. 1 presents a flow chart for the analysis of data obtained from heat inactivation studies (all symbols are as defined in Chapter 1). The reaction order for the inactivation process (n) has to be determined first before the rate constant of inactivation (k) can be estimated.

3. Results and Discussion

Fig. 2 shows comparisons of residual activity versus heating time for cod pepsin, cod pepsinogen and porcine pepsin at 55°C at pH 2 (full profile is not shown for porcine pepsin). The plot shows that cod pepsinogen was less stable than cod pepsin and porcine pepsin at pH 2. Linear regression (LR) analysis of these results showed a better agreement with the 1-st order model compared to a 2-nd order model. The activation parameters were then calculated from k values at different temperatures as described in Section 2.2.2.

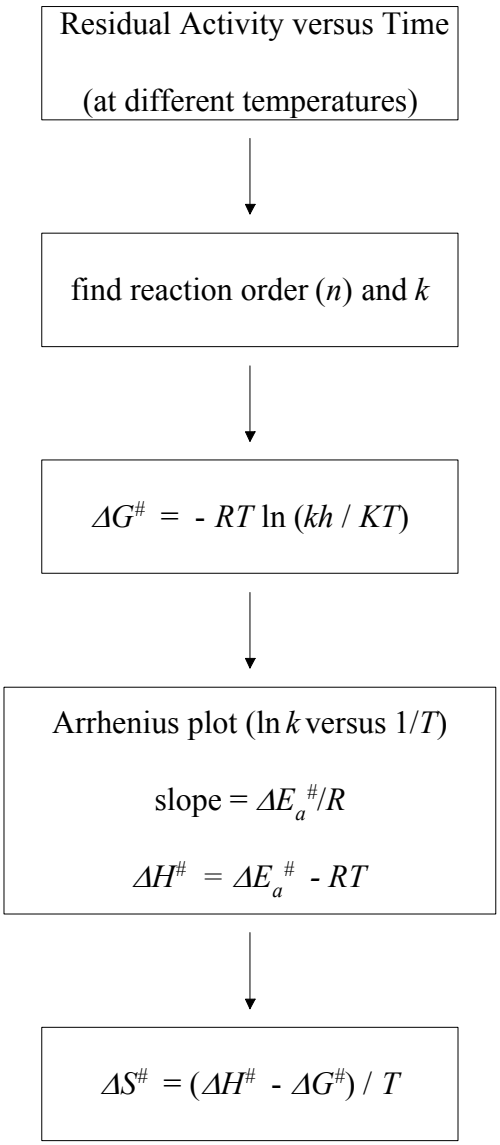

Fig. 1: Flow chart for analysis of data from heat inactivation studies.

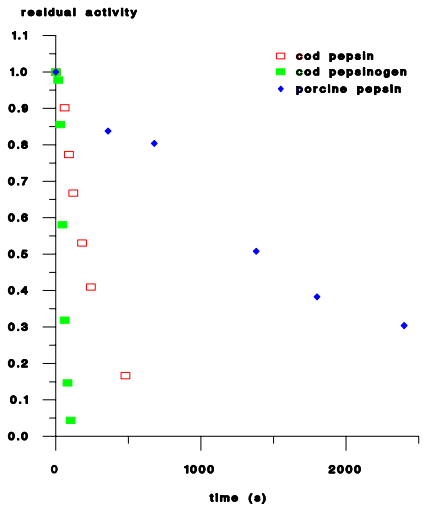

Fig. 2: Residual activity versus time at 55°C, pH 2.

Table 1(A,B, and C) lists the activation parameters for the heat inactivation of cod pepsinogen (CPG), cod pepsin (CP) and porcine pepsin (PP).

Table 1A: $\Delta G^{\#}$ for cod pepsinogen (CPG), cod pepsin (CP) and porcine pepsin (PP)

Temperature (°C)	$\Delta G^{\#}$ (kJ mol^{-1})		
	CPG	CP	PP
40	105	-	-
45	99	104	-
50	94	-	107
55	90	96	103
60	-	-	102
65	-	-	99

Table 1B: $\Delta H^{\#}$ for cod pepsinogen (CPG), cod pepsin (CP) and porcine pepsin (PP)

Temperature (°C)	$\Delta H^{\#}$ (kJ mol^{-1})		
	CPG	CP	PP
40	408	-	-
45	408	255	-
50	408	-	272
55	408	263	272
60	-	-	272
65	-	-	272

Table 1C: $\Delta S^{\#}$ for cod pepsinogen (CPG), cod pepsin (CP) and porcine pepsin (PP)

Temperature (°C)	$\Delta S^{\#}$ (J mol^{-1})		
	CPG	CP	PP
40	970	-	-
45	974	802	-
50	972	-	514
55	969	802	516
60	-	-	513
65	-	-	516

As mentioned before, $\Delta G^{\#}$ values indicate the activation free energy barrier to enzyme inactivation. Based on $\Delta G^{\#}$ values, the order of stability is porcine pepsin > cod pepsin > cod pepsinogen. At 55°C, the ratio of k or $\Delta G^{\#}$ values yields a stability ratio for porcine pepsin : cod pepsin : cod pepsinogen of about 118:7:1. Cod

pepsin and pepsinogen were significantly less stable than porcine pepsin..

$\Delta S^{\#}$ is a measure of the total (enzyme, solvent) disorder change associated with the formation of a transition state or an activated complex ($N^{\#}$). $\Delta H^{\#}$ is the heat change for producing $N^{\#}$. The order of stability based on $\Delta S^{\#}$ values is the same as for $\Delta G^{\#}$, that is, porcine pepsin > cod pepsin > cod pepsinogen. Large positive $\Delta S^{\#}$ values indicates an unstable enzyme because a positive $\Delta S^{\#}$ values (disorder increase) encourages $N \rightarrow N^{\#}$ reaction.

However, the order of stability based on $\Delta H^{\#}$ values is cod pepsinogen > porcine pepsin > cod pepsin. From these studies, it was observed that large $\Delta H^{\#}$ values associated with instability, contrary to what is expected. Large $\Delta H^{\#}$ means that large number of bonds must be broken to form $N^{\#}$. Therefore, the important reason for heat instability of cod proteases seems to be very large values of $\Delta S^{\#}$.

This finding suggests that $N^{\#}$ for cod proteases have a more flexible and looser structures compared to their porcine counterparts. The difference in heat stability of Atlantic cod pepsin and porcine pepsin cannot be interpreted from differences in the hydrophobicity and potential number of disulphide bonds. The major cause of the higher stability of porcine pepsin may be a higher level of hydrogen bonding [6]. It is also possible that the carbohydrate residues in cod protease may cause improved flexibility and reduced stability due to interference with weak intramolecular proteins bonds [4].

The difference in stability between cod pepsin and cod pepsinogen might be due to differences in their structures. The pI of cod pepsinogen and cod pepsin are 6.5 and 4.5, respectively (Amiza and Owusu, 2002). Activation of pepsinogen into pepsin is initiated by H^{+}. During activation, an activation segment is cleaved from the N-terminal of pepsinogen. In this study, cod pepsinogen was placed into heated pH 2 buffer (40-55^{o}C). It is possible that at such high temperature, the structure of cod pepsinogen was partially destroyed before it was activated. If cod pepsinogen had been first activated into cod pepsin before heat inactivation, then its stability would have been the same as determined for cod pepsin.

4 Conclusion

Cod pepsin and pepsinogen were significantly less stable than porcine pepsin at pH 2 in temperature range of 40-65^{o}C.

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