Protease Stabilization by Carboxylic Acid Salts: Relative Efficiencies and Mechanisms

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Kinetic studies are presented on the inhibition of proteolysis by carboxylic acid salts on the synthetic substrate succinyl ala-ala-pro-phe-para nitroanilide. The inhibition of proteolysis/autodigestion is shown to be the major factor in the stabilization of a detergent protease [i.e., Maxatase (subtilisin Carlsberg)] in an unbuilt, liquid, heavy duty laundry formulation. The inhibition of autodigestion by a carboxylic acid salt as a function of pH parallels the protease stability increase during accelerated aging. Relative inhibition of proteolysis and protease stabilization by various carboxylic acid salts are compared.

The use of enzymes in United States laundry detergents has experienced a renaissance since 1980. Over half the detergents sold in America now contain enzymes. Liquid laundry detergents have also experienced a tremendous increase in volume. Greater than 40% of all U.S. laundry detergents are liquid, and most of these liquid laundry detergents contain enzymes.

Until relatively recently, the inclusion of a detergent protease in a liquid laundry detergent was a technical impossibility, due to the enzyme's instability in the liquid formulation. In recent years, the patent literature has been filled with "enzyme stabilizers" that have overcome this instability problem (1-18). Though impressive in volume, the literature has not provided a mechanistic explanation of the phenomenon of protease stabilization in a liquid laundry detergent. The loss of enzyme activity during aging in a liquid laundry detergent. The loss of enzyme activity during aging in detergent formulations traditionally has been classified as protein denaturation, a first order process. Though it is true that stability data (percent initial enzyme activity vs time) can be adequately described by a semilog plot, which is indicative of a first order process, upon closer investigation, stability data is more completely described by a combination of first and second order processes. This study presents the results of an investigation into the mechanism of protease stabilization by short chain carboxylic acid salts in a high water content, unbuilt liquid laundry detergent.

EXPERIMENTAL

Stability studies. Maxatase LS 400,000 detergent protease (subtilisin Carlsberg), containing 400,000 Delft protease Units (DU) per gram (90% purity), was formulated into the unbuilt liquid heavy duty laundry detergent (LHDLD) shown in Table 1, then aged at 43.3°C, a temperature chosen to simulate "stress testing" of the enzyme stability analogous to that experienced by an enzyme containing liquid laundry detergent stored in a hot warehouse. Samples were withdrawn at 0, 7 and 14 days and protease activity determined using a para nitroanilide (pNA) assay (19). Experiments and data points were each done in triplicate. Enzyme assays described above had a precision of ± 2% of the amount present.

TABLE 1

LHDLD Formulation

Ingredient	Wt %
Neodol 2.3-6.5 (ethoxylated alcohol)	15
Neodol 25-3S (ethoxylated alcohol sulfate)	4
Triethanolamine	2
Ethanol	5
Calcium (free)	10 ppm
Maxatase LS 400,000[a]	1
Perfume, color, water	Q.S. 100%

[a]A proteolytic enzyme solution containing 400,000 Delft Units per gram manufactured by International Bio-Synthetics B.V., Rijswijk, Holland. Formulation pH's were adjusted with HCl.

TABLE 2

Inhibition Study Conditions

Temperature	43.3°C
Enzyme concentration	1 DU/ml
Substrate	succinyl-L-ala-ala-pro-phe para nitroanilide
Substrate concentrations	2.368 mM 1.184 mM 0.592 mM 0.296 mM 0.148 mM 0.074 mM 0.037 mM
Buffer	100 mM TRIS 20 mM sodium thiosulfate 10 mM calcium chloride pH adjusted with HCl

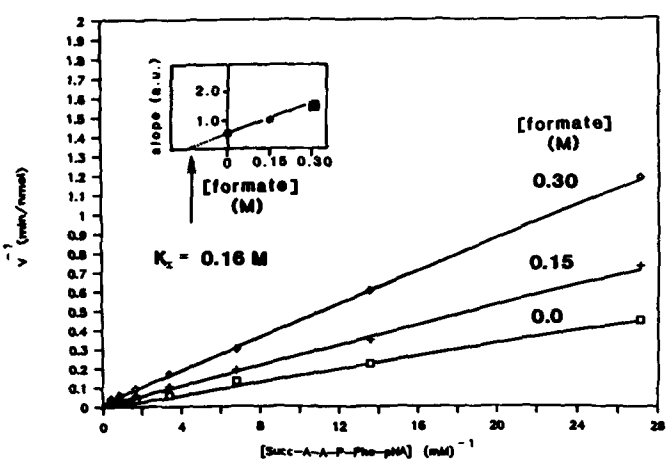

FIG. 1. Lineweaver-Burke plot of Maxatase protease. Substrate, ala-ala-pro-phe-para nitroanilide; temperature, 43.3°C, pH 7. Inset: Replot of the slopes from the Lineweaver-Burke plot to obtain k_I.

The free calcium concentration of the formulations was adjusted to be 10 ppm by taking into account the Ca^{++} binding of the formula constituents. The dependence of enzyme stability on two variables, formulation pH and Na carboxylate concentration, was investigated in this study. Formulas were prepared with 0, 1, 2 and 3% sodium carboxylate (formate, acetate and propionate) at formulation pH's from 6.0 to 8.0. Thus, an entire profile of isostability profiles vs pH and sodium carboxylate concentration for formate, acetate and propionate, under accelerated aging conditions, was obtained.

Inhibition studies. Inhibition studies were performed using a variation of the pNA assay. Assay conditions are presented in Table 2. Assay conditions were selected to be analogous to those in the accelerated aging studies. Inhibition of proteolysis as a function of pH and carboxylate concentration was determined. For clarity, an example of the data is presented as a Lineweaver-Burke plot (inverse rate vs inverse substrate concentration, Fig. 1). Inhibitor binding constants (K_I) and type of inhibition were determined from Eadie-Hofstee plots.

Kinetic analysis. Once protease stability profiles (percent initial activity as a function of time) were known for the liquid laundry detergent formulation (Table 1) as a function of pH and carboxylate concentration, and K_I was known as a function of pH, the stability data was fit to a proposed mechanism (see below).

REACTION SCHEMES

$$E_a \xrightarrow{k_1} E_d \qquad \text{denaturation}$$

$$E_a + E_a \xrightarrow{k_2} E_aE_a \xrightarrow{k_3} E_a + E_d \qquad \text{autodigestion}$$

$$E_a + I \underset{}{\overset{K_{I1}}{\rightleftharpoons}} E_aI \qquad \text{inhibition}$$

$$E_aE_a + I \overset{K_{I2}}{\rightleftharpoons} E_aE_aI \qquad \text{inhibition}$$

$$K_{I1} = \frac{(E_a)(I)}{(E_aI)}$$

$$K_{I2} = \frac{(E_aE_a)(I)}{(E_aE_aI)}$$

$$E_aI_a \xrightarrow{k_4} E_d + I \qquad \text{denaturation}$$

$$E_aI + E_a \xrightarrow{k_5} E_d + I + E_a \qquad \text{autodigestion of } (k_5)/\text{by } (k_6) \text{ the enzyme inhibitor complex}$$

$$E_aI + E_a \xrightarrow{k_6} E_aI + E_d$$

Where E_a is an active enzyme and E_d is a denatured or autodigested, inactive enzyme, E_aE_a is an enzyme-substrate complex, E_aI is an active enzyme-inhibitor complex and E_dI is a denatured or autodigested enzyme inhibitor complex.

It was assumed that the rate limiting step in the autodigestion of the protease was the formation of the enzyme-substrate complex, E_aE_a, and that the concentration of E_aE_a was always very small and the inhibitor binding constant for the enzyme E_a was the same as for the complex (i.e., $K_{I1} = K_{I2}$). The second order rate constant k_2 is therefore defined as the overall rate of E_d formation through autodigestion. Under these assumptions, the reaction scheme presented above simplifies to that presented below.

REACTION SCHEMES

$$E_a \xrightarrow{k_1} E_d \qquad \text{denaturation}$$

$$E_a + E_a \xrightarrow{k_2} E_a + E_d \qquad \text{autodigestion}$$

$$E_a + I \underset{}{\overset{K_I}{\rightleftharpoons}} E_aI \qquad \text{inhibition}$$

$$K_I = \frac{(E_a)(I)}{(E_aI)}$$

$$E_aI \xrightarrow{k_3} E_d + I \qquad \text{denaturation}$$

$$E_aI + E_a \xrightarrow{k_4} E_d + I + E_a \qquad \text{autodigestion of } (k_4)/\text{by } (k_5) \text{ the enzyme inhibitor complex}$$

$$E_aI + E_a \xrightarrow{k_5} E_aI + E_d$$

Where E_a is an active enzyme and E_d is a denatured or autodigested, inactive enzyme, and E_aI is an active enzyme-inhibitor complex and E_dI is a denatured or autodigested enzyme inhibitor complex.

The simplified scheme was the one used for data analysis.

k_1 (first order denaturation constant) was determined at each pH by assuming that the protease instability was exclusively a first order process and plotting k_1 vs 1/(carboxylate) and extrapolating to 1/(carboxylate) = 0. The y axis intercept (at infinite carboxylate concentration) was taken as k_1, the denaturation rate constant without the complication of proteolytic autodigestion. k_1 was determined at each pH and independently for formate, acetate and propionate. The k_1's obtained for each of the carboxylic acid salts were identical within experimental error.

In the reaction scheme, a(t), b(t), c(t), d(t) and e(t) are taken to represent the time dependent increments of concentration, since the beginning of aging of the species $E_a^{(1)}$ [from denaturation decay (i.e., k_1)], $E_a^{(2)}$ [from autodigestion of E_a by E_a (i.e., k_2)], $E_aI^{(1)}$ [from denaturation decay of the E_aI complex (i.e., k_3)], $E_aI^{(2)}$ [from autodigestion of the E_aI complex by E_a (i.e., k_4)] and $E_aI^{(3)}$ [from autodigestion of E_a by the E_aI complex (i.e., k_5)], respectively. The initial species concentrations after mixing and before aging were calculated using the K_I obtained from the inhibition studies.

The time dependent concentrations of $E_a^{(1)}$ (thermodynamic decay), $E_a^{(2)}$ (autodigestion), [$EaI^{(1)}$] (thermodynamic decay), [$EaI^{(2)}$] (autodigestion), and $E_aI^{(3)}$ (autodigestion of E_a by E_aI) are therefore given by (E_a-a), (E_a-b), (E_aI-c), (E_aI-d) and (E_aI-e), respectively.

Concentrations of each species were updated throughout the calculation, using the calculated rate constants and K_I obtained from the inhibition experiments.

The differential equations for the kinetics are:

$$dE_a/dt = -k_1 (E_a - a)$$
$$dE_a/dt = -k_2 (E_a - b)^2$$
$$d(E_aI)/dt = -k_3 (E_aI - c)$$
$$d(E_aI)/dt = -k_4 (E_aI - d) (E_a)$$
$$d(E_aI)/dt = -k_5 (E_aI - e) (E_a)$$

Numerical integration was performed using the Runge-Kutta method (20-21).

For all of the cases described by the figures, the continuous curves were computed with the aid of the rate constants derived from the mathematical treatment of the differential equations. The experimental data at each pH for all the carboxylate types and concentrations was analyzed according to the reaction scheme above. A statistical analysis was made to justify the inclusion and independence of each of the rate constants in the final proposed mechanism.

RESULTS AND DISCUSSION

Inhibition studies. An example of results from an inhibition experiment is presented as a Lineweaver-Burke plot in Figure 1. Only experimental results performed using Na formate at pH 7 are shown, for simplicity. In this experiment, the effect of Na formate concentration on the rate of proteolysis (graphed as inverse proteolysis rate vs inverse substrate concentration) at pH 7 is presented. In the inset, a replot of the data is shown (slope of the Lineweaver-Burke plot vs formate concentration). K_I, the inhibitor binding constant, is obtained from the x axis intercept of the replot.

All data generated from the inhibition experiments may be explained by noncompetitive inhibition of the protease by the carboxylic acid salt. This type of inhibition is classified by a decrease in the velocity of the enzyme without any change in the affinity of the enzyme for its substrate. This is consistent with the carboxylic acid salts binding at a position away from the enzyme active site.

A summary of the inhibitor binding data as a function of pH for formate, acetate and propionate is presented in Figure 2. As pH increases the K_I also increases, indicative of weaker binding of the carboxylic acid salts by the enzyme and thus of less inhibition of proteolysis and autodigestion. This data may also be explained by noncompetitive inhibition of the protease exclusively by the carboxylic acid and not by the carboxylate. The increase in K_I with pH is due to the decreasing concentration of the acid as the pH gets farther from the pK_a of the acid.

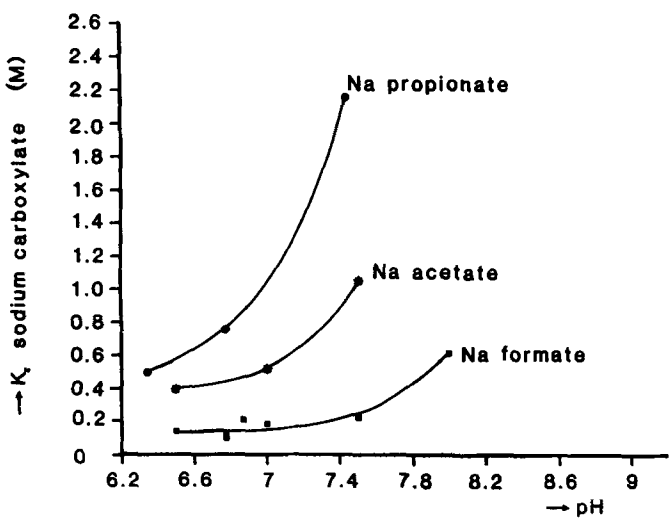

FIG. 2. Calculated K_I's for Na formate, Na acetate and Na propionate for Maxatase as a function of pH. Temperature, 43.3°C.

At pH's above 8 with sodium formate, greater than 0.6 molar, or 4% formate, is required to reach K_I and thus reduce the concentration of active, uninhibited enzyme by 50%. However, at lower pH's, for example, pH 7, K_I is only 140 mM, or 1% Na formate, a reasonable concentration in a liquid laundry detergent.

At all pH's investigated, the effectiveness of inhibition by short chain carboxylic acid salts was formate > acetate > propionate. As pH increases, the relative inhibition of formate versus acetate or propionate increases as well.

Enzyme stability studies. The stability of 1% Maxatase LS 400,000, a detergent protease, in the unbuilt, unstabilized, pH 7 liquid laundry detergent formulation under accelerated aging conditions at 110°F, is presented in Figure 3. The inclusion of 1% sodium formate produces a dramatic increase in protease stability; however, the same molarity of sodium chloride (0.85%) produces no stability increase.

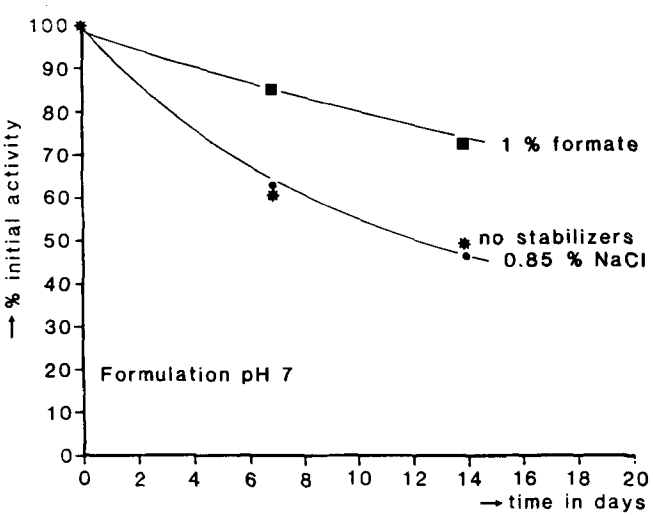

FIG. 3. Stability of Maxatase at 43.3°C in a high water content liquid HDLD with stabilizer (1% Na formate) ■, without stabilizer * or with 0.85% NaCl added •. Rate constants used to calculate draw curves, $k_1 = 9.84 \times 10^{-8}$ sec $^{-1}$ and $k_2 = 0.055$ m^{-1} sec^{-1}.

The effect of sodium formate concentration on protease stability in an unbuilt liquid HDLD is presented in Figure 4, where the half life of Maxatase is plotted as a function of the sodium carboxylate concentration in the laundry detergent formulation. Increasing the sodium formate concentration produces an increase in stability, but one does not obtain complete stability even at high formate concentrations. Continuous curves were generated using calculated half-lives obtained from a solution of the differential equations. Data points were obtained from accelerated aging stability studies at 110°F.

A comparison of enzyme stabilization by sodium for-

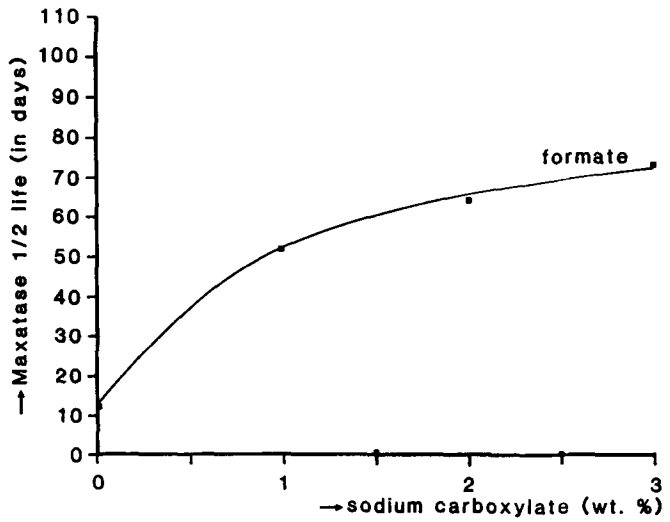

FIG. 4. Maxatase ½-life in a high water content liquid HDLD at 43.3°C as a function of Na formate concentration.

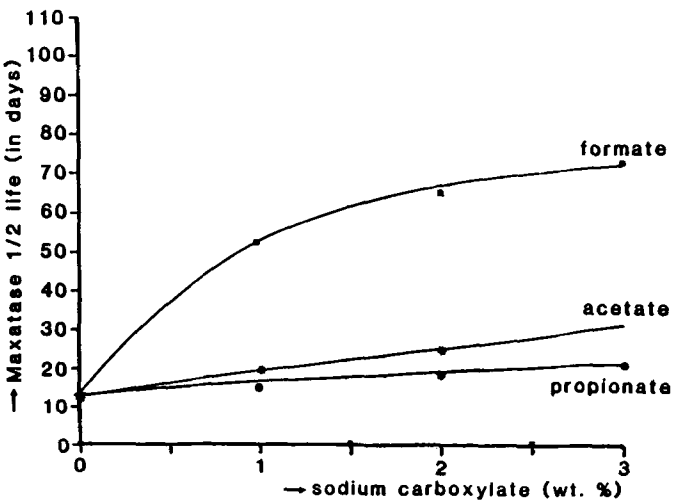

FIG. 5. Maxatase ½-life in a high water content pH 7 liquid HDLD at 43.3°C as a function of Na formate, Na acetate and Na propionate concentration.

mate, acetate and propionate on a weight basis is presented in Figure 5. Relative stabilization effectiveness may be ranked formate > acetate > propionate on both a mole and weight basis, analogous to the order of efficacy in noncompetitive inhibition.

The instability of a protease in a liquid laundry detergent is due to a combination of processes. Within the precision of the stability data, the stabilization of Maxatase by carboxylic acid salts in a high water content, liquid laundry detergent between pH 6.0 to 7.5 can be explained by a combination of first order denaturation, second order proteolytic autodigestion and noncompetitive inhibition of proteolysis by the carboxylic acid salts. Stability data at pH 8.0 could not be fit with this mechanism; probably due to the decrease in quality of the K_I's determined at this high a pH.

Traditionally, the instability of a protease in a liquid laundry detergent has been explained by first order denaturation of the protease. Such a simplistic explanation of protease instability offers no explanation of the stabilization mechanism of carboxylic acid salts. A comparison of the quality (variance) of a first order fit for an individual experiment (i.e., pH and carboxylic acid salt concentration) is actually superior to that provided by the scheme suggested here. However, a first order rate constant (k_1) must be calculated for each pH and carboxylic acid salt concentration. This is in contrast to the proposed scheme where the first order (k_1) and second order (k_2) rate constants are fixed for each pH and the increased stability observed upon the addition of carboxylic acid salts is provided by the noncompetitive inhibition of autodigestion.

A more relevant comparison of the first order scheme versus the noncompetitive inhibition scheme presented here would be obtained by comparing the quality of fits for each scheme in a plot such as Figure 4 which describes the effect of increasing carboxylate concentration on protease stability. No such comparison can be made, because the first order scheme does not predict any stability increase.

TABLE 3

Calculated Rate Constants

pH	k_1 (sec^{-1})	k_2 (M^{-1} sec^{-1})
6.5	7.52×10^{-8}	0.0446
7.0	9.84×10^{-8}	0.0550
7.5	2.60×10^{-7}	0.0790

Within the precision of the analysis, one may draw the following conclusions:

- The enzyme-inhibitor complex (E_aI) denatures at the same rate as the uninhibited enzyme (E_a) (i.e., $k_1 = k_3$).
- The enzyme inhibitor complex (E_aI) is autodigested at the same rate as the uninhibited enzyme (E_a) (i.e., $k_2 = k_4$).
- The enzyme inhibitor complex (E_aI) has no proteolytic (autodigestive) activity (i.e., $k_5 = 0$).

Rate constants calculated from the stability data are presented in Table 3. Under the conditions of this experiment, the second order rate term (autodigestion) dominates the proteolytic activity loss during most of the aging. Thus, inhibition of autodigestion produces an effectively stabilized enzyme system in this formulation type.

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