Enzymic Activity of Proteases in Detergent Systems: Comparison of Assay Methods and the Role of Interfering Substances

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Abstract

Two modifications of a method are presented for assaying proteolytic enzymes. They differ primarily in the mechanism used for terminating the enzymic reaction, one technique employing trichloroacetic acid (TCA), the other, heat inactivation. Comparative studies reveal that there are two distinct disadvantages in using TCA which are not evident with the heat inactivation procedure. In the first place, TCA does not selectively precipitate residual substrate. Secondly, in the presence of acid, linear alkyl benzene sulfonate and other long chain anionic surfactants interfere with the quantitative determination of enzymic activity. This interference can be falsely interpreted as inhibition of proteolysis. An explanation of the above phenomena is given in the text along with assay results in the presence of metal chelating agents.

Introduction

The recent emergence of hydrolytic enzymes as additives to laundry detergents has resulted in an extensive re-examination of the biochemical properties of these proteins and the organisms that produce them. Most of the emphasis has been centered on proteases derived from various strains of *Bacillus subtilis* and the bioassays used to measure their activity. Since the combination of enzymes and household detergents is a relatively new endeavor, little information is available in the literature. Some controversy and much conjecture have arisen during the investigation of the effect of certain detergent ingredients, such as the surfactant and the phosphate builder, on the activity of enzymes.

Most proteolytic assays are based on the hydrolysis of a protein substrate followed by termination of the reaction with trichloroacetic acid (TCA), (e.g., Anson, Delft, etc.). TCA prevents further enzymic reaction by lowering the pH and precipitating residual substrate and enzyme. Quantitative determination of the enzyme's activity is performed by measuring the amount of digested substrate remaining in the acid soluble fraction. A number of reports (1–5) have implicated anionic detergents as inhibitors of enzymic activity. In contrast, it has been stated (6) that in general, a bacterial protease is compatible with all types of detergents, but some detergents interfere with the assay procedure rather than inhibiting enzymic activity. Although anionic detergents have been the main subject of the conflicting reports, there also exists some disagreement on the inhibitory effect of sodium tripolyphosphate (4–6).

In this report we present a comparison of results obtained using two bioassay procedures with different means for terminating the enzymic reaction. In one procedure TCA is utilized and in the other heat denaturation of the enzyme is employed. An explanation for the discrepancy between the results of the two procedures is offered showing the advantage in avoiding the use of TCA especially in the presence of anionic detergents. The heat denaturation method is further used to study the effects of anionic detergents and chelating agents on enzymic activity.

Materials and Methods

Two modifications of a proteolytic assay were used for determining enzyme potency. They vary in one main respect, the method for terminating the enzymic reaction. This variation, although minor in execution, results in a major difference in the ultimate result obtained. In one case, acid is used to terminate the reaction and analyses are performed on the supernatant fraction. In the second method, heat is used to terminate the reaction and enzymic activity is measured using the complete reaction mixture. In both cases, activity is ultimately determined after substrate digestion by using ninhydrin reagent to determine the amino groups produced by hydrolytic cleavage of peptide linkages.

Materials

Ammediol buffer. (a) 2-Amino-2-methyl-1,3-propanediol: 21.3 g/L; (b) 0.1 N HCl. For pH 9.0 at 50 C, add 1000 ml of (a) to 500 ml of (b) and dilute to 4000 ml with water.

Casein (Hammersten) Solution. Prepare a solution of 0.5% w/v in ammediol buffer. Heat for 10 min. in a boiling water bath to solubilize.

Citrate buffer. (a) Citric Acid (monohydrate): 21.01 g/500 ml; (b) Trisodium citrate: 29.41 g/500 ml. For pH 5.1, add 20 ml of (a) to 55 ml of (b) and dilute to 100 ml with water.

Ninhydrin (1,2,3-Indantrione Monohydrate) Solution. Dissolve 2 g of ninhydrin in 300 ml of 1.3×10^{-4} M KCN in peroxide-free methyl cellosolve (ethylene glycol monomethyl ether) solution.

Trichloroacetic Acid. Prepare a solution of 5% w/v in deionized water.

Commercial Protease A. Alkaline type.

Commercial Protease B. Neutral type.

Commercial Protease C. Primarily neutral type but with a significant amount of alkaline protease.

Crystalline Neutral Bacterial Proteinase. A crystalline material from a strain of *B. subtilis* which produces a mixture of neutral and alkaline proteases. 25.9 Anson units/g. Novo Industri, Copenhagen, Denmark.

Crystalline Alkaline Bacterial Proteinase. A crystalline material from a strain of *B. subtilis* which produces primarily alkaline protease. 26.0 Anson units/g. Novo Industri, Copenhagen, Denmark.

Constant temperature water baths.

TABLE I
Comparison of Results; Proteolytic Activity of Protease A (alkaline type—15 μg/ml) by TCA Precipitation and Heat Inactivation Assays

Incubation time (min)	Absorbance (A)		A TCA–A heat
	A[a] heat	A[a] TCA	
10	0.20	0.36	0.16
15	0.28	0.53	0.25
20	0.32	0.65	0.33
25	0.37	0.78	0.41
30	0.45	0.88	0.43
40	0.50	1.0	0.5
50	0.67	1.2	0.5
60	0.75	1.4	0.7
75	0.78	1.5	0.7
Control	0.64[b]	0.03[b]	

[a] Increase in absorbance over the controls.
[b] Absorbance of controls versus buffer solution.

Bausch & Lomb Spectronic 20 spectrophotometer equipped with stationary transfer cell.

International portable refrigerated centrifuge, model PR-2.

Methods

Trichloroacetic Acid Precipitation. In the assay, 1.0 ml of an appropriate concentration of enzyme or enzyme-detergent solution in ammediol buffer is added to 1.0 ml of casein (in triplicate). The enzyme is allowed to digest the casein at 50 C for a specified period of time (usually 15 min but the time can be varied depending on enzyme potency and purity). At the conclusion of the incubation period, 2.0 ml of TCA solution is added which terminates the reaction and precipitates undigested casein. The controls are treated in the exact manner as the test samples, except that enzyme is omitted.

The samples are allowed to stand for 30 min at room temperature followed by centrifugation at 2200 rpm to remove the TCA precipitated casein. Color development is accomplished by mixing 0.3 ml (or a suitable volume depending on enzyme potency) of the TCA supernatant liquid with 2.5 ml of citrate buffer and 1.5 ml of ninhydrin solution and immersing in a boiling water bath for 18 min. The samples are cooled and their absorbancies are measured in the colorimeter at 570 mμ with the controls serving as reference solutions.

Heat Inactivation. This method is similar to the preceding TCA precipitation method, except that heat denaturation is used to terminate the enzymic reaction. At the conclusion of the incubation period, samples are immersed in a water bath at 90 C for 5 min. In order to obtain identical volumes for the two methods for comparison purposes, 2.0 ml of water instead of TCA solution is added after heat inactivation. No centrifugation is necessary since the solution is homogeneous. Ninhydrin analyses of amino groups are performed on the total inactivated reaction mixture as described above. Controls are treated similarly except that enzyme is omitted.

Results and Discussion

Comparison of Assay Procedures in the Absence of Detergents

In the initial studies comparing the two assay procedures, proteolytic hydrolysis of casein was examined in the absence of any detergent material. A considerable difference was noted in the amount of ninhydrin reactive material remaining after inactivating the enzyme. Results using TCA were always higher than those obtained with heat inactivation.

TABLE II
The Effect of Heat Inactivation on Amino Groups of Casein Digests[a]

Sample	Absorbance	
	TCA precipitation	Heat inactivation-TCA post added
1	0.34	0.34
2	0.34	0.34
3	0.35	0.35
4	0.35	0.34
5	0.35	0.34

[a] Casein digestion was accomplished with neutral protease B.

A study comparing TCA precipitation to heat inactivation as a function of incubation time lends considerable insight as to the main differences between the two methods of assay. Results of such a study using the commercial protease A are shown in Table I.

As evident from the data presented in Table I, the reference casein control for the heat inactivated samples has a rather high optical density of 0.64 relative to buffer alone. The TCA counterpart has a low reading of 0.03 in comparison. In contrast, the enzymic digests obtained by heat inactivation are at all times lower in absorbance than the digests of the TCA systems.

The explanation for both of these phenomena is believed to be as follows. Ninhydrin developed color arises from two sources: amino groups originally present in the casein, and amino groups generated by enzymic hydrolysis of peptide bonds. The TCA-casein control develops little or no color because virtually all the casein is removed by precipitation. However, as enzymic digestion proceeds, low molecular weight fragments of the casein are produced which do not precipitate upon subsequent addition of TCA. Consequently, in the TCA procedure, a fraction of the original amino groups as well as newly generated groups remain in the supernatant liquid and produce color with ninhydrin. On the other hand, in the heat inactivated method, the original amino groups are not measured since the absorbance of the digest is taken relative to a reference solution containing all of the original casein substrate. Therefore, the absorbance is solely a measure of the new amino groups introduced by peptide bond cleavage. Thus, absorbancies in the TCA method are higher than those in the heat inactivation procedure, the difference in absorbancy between the two methods increasing with incubation time as more of the original amino groups appear in the TCA supernatant liquid. The difference in absorbance between the two methods approaches a constant value as enzymic digestion nears completion and all original amino groups remain in the TCA soluble fraction. This constant value corresponds closely to the 0.64 absorbance found for the control as shown in the preceding table. The conclusion is that the heat inactivation procedure is a more accurate measure of proteolytic hydrolysis since the amino groups arising from peptide cleavage alone are measured.

An experiment was run to determine if heat denaturation alters the amino group content of casein or causes hydrolysis of peptide bonds. The enzyme-casein reaction was terminated with TCA and analyses were compared with an identical set of samples which were heat inactivated, cooled and TCA was added only after cooling. Results of a typical experiment using protease B are shown in Table II.

From this study, it appears that the heating step does not in any way affect the amino group content.

Assay of Proteases in the Presence of Anionic Detergent

Initial data obtained in assaying proteolytic enzymes in the presence of linear alkyl benzene

TABLE III

Proteolytic Activity in the Presence of LAS using TCA Precipitation[a]

Test system	Absorbance	
	Exp. 1	Exp. 2
Enzyme in absence of LAS	0.53	0.51
Enzyme with LAS added initially	0.45	0.42
Enzyme wth LAS added at end of incubation period	0.49	0.41

[a] Neutral protease B.

sulfonate (LAS) and using TCA for reaction termination indicated that this anionic surfactant inhibited proteolytic activity. Absorbance values obtained from assaying an LAS-enzyme system were always lower than those obtained using an enzyme in the absence of LAS. When LAS was added just prior to reaction termination and compared to the same system in which the LAS was present during the entire incubation period, certain abnormalities were noted. The data obtained from this type of study with neutral protease B (55 μg/ml) and an LAS concentration of 205 μg/ml are shown in Table III.

It is evident from the data in Table III that LAS interferes mainly with the assay procedure after enzymic hydrolysis, since addition of LAS just prior to reaction termination with TCA also led to low results. It is postulated that the addition of TCA lowers the pH of the solution below the isoelectric point of the casein digest. The cationic (ammonium) form of the digest then interacts with the anionic surfactant and precipitation of a complex leads to a loss of some ninhydrin reactive material from the supernatant liquid. Evidence for this postulated interference caused by TCA was obtained in a series of experiments employing the heat inactivation method. In one set of studies, enzyme was assayed in the presence and absence of LAS, followed by heat inactivation and the addition of 2.0 ml of water. A comparative set of samples was treated in identical fashion except that after heat inactivation, 2.0 ml of 5% TCA solution was added. Results using protease B are shown in Table IV.

The data demonstrate that when heat inactivation is employed and no TCA added, LAS interference is absent. However, under identical conditions, the addition of TCA leads to a definite interference by LAS. This experiment confirmed previous indications that LAS was not inhibiting the enzyme but merely interfering with measurable end products in the presence of TCA. Crystalline enzymes yielded similar results.

Experiments also showed that LAS did not interfere with ninhydrin color development. This was tested by examining the color development of various casein digests (e.g., trypticase, casitone, etc.) in the presence and absence of LAS. No differences in absorbance readings were observed.

To further clarify the role played by LAS in assay interference, lower molecular weight homologues of LAS were investigated using the TCA precipitation

TABLE IV

Proteolytic Activity in the Presence of LAS Using Heat Inactivation[a]

	Absorbance			
	No TCA		TCA	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Enzyme & LAS	0.14	0.13	0.17	0.17
Enzyme alone	0.12	0.11	0.23	0.26

[a] Neutral protease B.

TABLE V

Proteolytic Activity in the Presence of LAS or Lower Molecular Weight Homologues (TCA Procedure)

System[a,b]	Absorbance at 570 mμ	
	Exp.1	Exp. 2
Enzyme + LAS	0.46	0.40
Enzyme + Na xylene sulfonate	0.63	0.60
Enzyme + Na cumene sulfonate	0.69	0.61
Enzyme alone	0.64	0.60

[a] Assay concentrations of LAS, Na Xylene Sulfonate, and Na Cumene Sulfonate were 5×10^{-4} M.
[b] Neutral protease B.

method. Homologues tested were sodium xylene and cumene sulfonates. Results of two individual studies using protease B are shown in Table V.

As indicated by the results in Table V, low molecular weight homologues of LAS, as exemplified by the xylene and cumene sulfonates, do not lead to low absorbance values as in the case of LAS. This indicates that the alkyl chain plays a role in the detergent-protein interaction leading to apparently lower activity. A similar trend was reported by Karush and Sonenberg (7) who found increased affinity of bovine serum albumin for alkyl sulfate homologues of increasing chain length. Other long chain anionic surfactants, e.g., tallow alcohol sulfate and alkenyl sulfonate, also gave low results in the TCA procedure. On the other hand, long chain nonionic surfactants did not interfere with the method. These data support the previously stated contention that the ammonium form of the digest interacts with the anionic surfactant causing low results. Similar results were obtained when protease A was used.

From the above data then, there appear to be two major disadvantages to the use of TCA for terminating the enzymic reaction and removing residual substrate. In the first place, even in the absence of anionic surfactants, the use of TCA as a precipitant, as we have shown previously, cannot selectively differentiate between the amino groups originally present and the newly formed groups from peptide hydrolysis. Secondly, in the presence of anionic surfactants, a complex is formed with the cationic form of the casein digest, and precipitation of the complex causes low results which previously had been ascribed to enzyme inhibition by surfactant. The method employing heat inactivation reported in this publication is not affected by these two factors.

Enzymic Reactions in the Presence of Chelating Agents

While studies reported thus far have been mainly concerned with the effect of LAS (one of the active ingredients of detergent formulas) on the enzymic reaction, several experiments were performed using tripolyphosphate (TPP) both alone and in combination with LAS. It was noted in several instances, that some enzymes were affected by the TPP whether the TCA or heat inactivation method was employed. A pattern began to develop in that so-called alkaline proteases were not appreciably affected by TPP

TABLE VI

Proteolytic Activity in the Presence and Absence of TPP[a]

Incubation time (min)	Absorbance			
	Protease A + TPP	Protease A alone	Protease C + TPP	Protease C alone
5	0.11	0.11	0.07	0.11
10	0.17	0.17	0.12	0.17
15	0.24	0.22	0.17	0.27
25	0.33	0.36	0.25	0.31
35	0.41	0.46	0.28	0.42

[a] Assay concentration of TPP: 0.53 mg/ml; activity was determined for both protease A (alkaline type) and protease C (primarily neutral type).

TABLE VII

Proteolytic Activity in the Presence of EDTA[a]

Molar EDTA	Absorbance	
	Alkaline protease[b]	Neutral protease[b]
0.01	0.31	0.11
0.001	0.29	0.08
0.0005	0.30	0.12
0.0001	0.41	0.23
0	0.45	0.38

[a] Trisodium salt.
[b] Assay Concentration: 2.0 μg/ml.

whereas neutral proteases were inhibited. Both crude and crystalline enzymes generally followed this pattern even though the crude materials were not completely of the alkaline or neutral type. Typical data are shown in Table VI using the heat inactivation method and protease A (alkaline type) and protease C (primarily neutral type).

The inclusion of LAS with the TPP did not appreciably alter the result obtained with TPP by itself.

While TPP did seem to affect neutral protease activity, a more dramatic result was obtained when the more powerful chelating agent EDTA was employed. In this set of studies crystalline enzymes were used and the data obtained are shown in Table VII.

The activity of both crystalline enzymes is decreased in the presence of EDTA. The neutral enzyme, however, is more severely affected. A similar result has been reported by Hagihara (8). In both cases, there appears to be concentration at which additional amounts of EDTA are not harmful. The above phenomena may be due to the requirement of a metal co-factor by the neutral enzyme for maximum activity. The metal chelating agents, TPP and EDTA, lower the metal ion activity in solution, and therein possibly decrease the rate of enzymic hydrolysis. Alternately, EDTA may affect the alkaline protease by sequestering calcium, a metal not necessarily required for activity, but required for structural stability of the enzyme (9).

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