Automated Bioassay of Proteolytic Enzymes in Detergents[1]

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Abstract

An automated biological assay for proteases in detergents was developed. The assay involved enzymic digestion of casein followed by the analysis of the liberated amino groups. Quantitative determination of the proteolytic activity was accomplished by reacting the amino groups with trinitrobenzene-sulfonic acid (TNBS). An automated system was used to carry out both the digestion and the quantitative color development of the TNBS-amino group complex. The method is accurate, reproducible, easy to handle, fast and free from interferences by any of the standard detergent ingredients. It may also be used as an investigative tool in enzyme research.

Introduction

The combination of enzymes and detergents in commercial products has just emerged in this country. Relatively little information is available in the literature with regard to the analysis of hydrolytic enzymes in the presence of detergents. Efforts to study the biochemical properties of these enzymes and the possible effect of certain detergent ingredients on their activity necessitated a reliable assay procedure. The present work describes the successful development and application of an automated biological assay to quantitatively determine the activity of enzymes in formulated products.

Experimental Procedures

Apparatus

Technicon AutoAnalyzer System: Sampler II with various speed cams; proportioning pump; heating bath, controlled temperature, two 40 ft glass coils; colorimeter, 15 mm flow cell, 0.6 aperture; and recorder.

Materials

Casein (Hammersten): 0.5% w/v in buffer. The appropriate amount of casein is dispersed in buffer, heated to the boil, and cooled to room temperature. The solution is filtered through No. 41 filter paper and refrigerated when not in use. The solution is stable for four or five days.

Buffer. Five hundred milliliters of 0.2 M KCl, 500 ml of 0.2 M boric acid, 472 ml of 0.2 M NaOH, and 528 ml H_2O are mixed for a final pH of 9.4 at room temperature.

Trinitrobenzene-sulfonic Acid. (Nutritional Biochemicals Co., Cleveland, Ohio). Trinitrobenzene-sulfonic acid (TNBS) 0.1% w/v in water is kept in a dark bottle and refrigerated when not in use. With time, it changes from almost colorless to a definite yellow. This darker solution causes a change in the baseline, but this change is easily compensated for by the recorder.

Sample Preparation

Samples of finished product must be chosen by a suitable sampling technique as illustrated in Figure 1. The box of finished product is placed without shaking on its flat side. The face of the box is then cut diagonally with a sharp knife and the four parts moved back. A soup spoon or similar scoop device is used to obtain several portions of the sample at various places in the box, thus assuring a representative sample. The samples are transferred to pint-size Mason jars which may then be fitted on an Osterizer for grinding. This procedure eliminates a dust problem as well as the possibility of error in transferring samples and the possible pick up of moisture. A 500 mg sample is weighed out on an analytical balance and diluted with borate buffer to a 50 ml volume in a volumetric flask. The solution is thoroughly mixed, filtered through No. 41 filter paper, and approximately 2 ml of the filtrate are transferred to a small plastic cup which fits on the sampler tray of the AutoAnalyzer.

Chemical Analysis

The automated procedure for analysis is represented schematically in Figure 2. Buffer solution is continuously pumped into the reservoir on the back of the sampler. The aspirated sample, always separated from the next sample by a cup filled with buffer, is mixed with casein and segmented with air before going into the heating bath. After an appropriate incubation period (generally 14 min) at 50 C, the digested mixture is diluted with water, mixed and an aliquot is taken for analysis; the remaining solution is discarded. The aliquot is mixed with buffer, TNBS, segmented again with air, and returned to the second coil in the heating bath for

[1] Presented at the AOCS Meeting, New York, October, 1968.

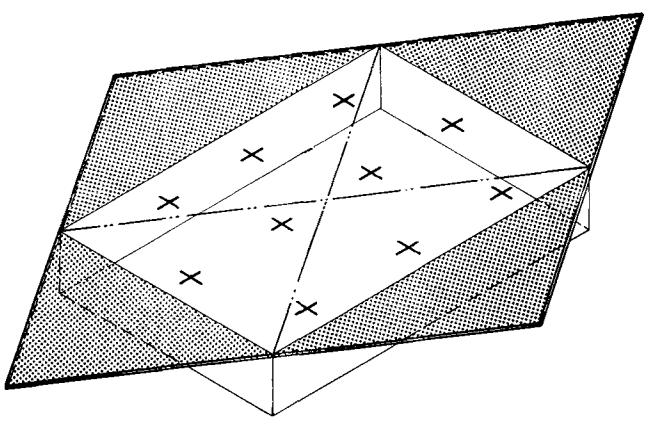


FIG. 1. Sampling technique.

TABLE I
Comparison of Methods
Weight Per Cent of Enzyme Found

Sample	Manual Method		Automated Method
	Ninhydrin	TNBS	TNBS
A	0.21	0.20	0.20
B	0.34	0.31	0.32
C	0.39	0.39	0.42
D	0.41	0.45	0.42
E	0.52	0.46	0.48
F	0.59	0.59	0.60
G	0.62	0.52	0.55
H	0.64	0.57	0.59
I	0.73	0.60	0.61
J	0.75	0.70	0.73
K	0.75	0.72	0.72

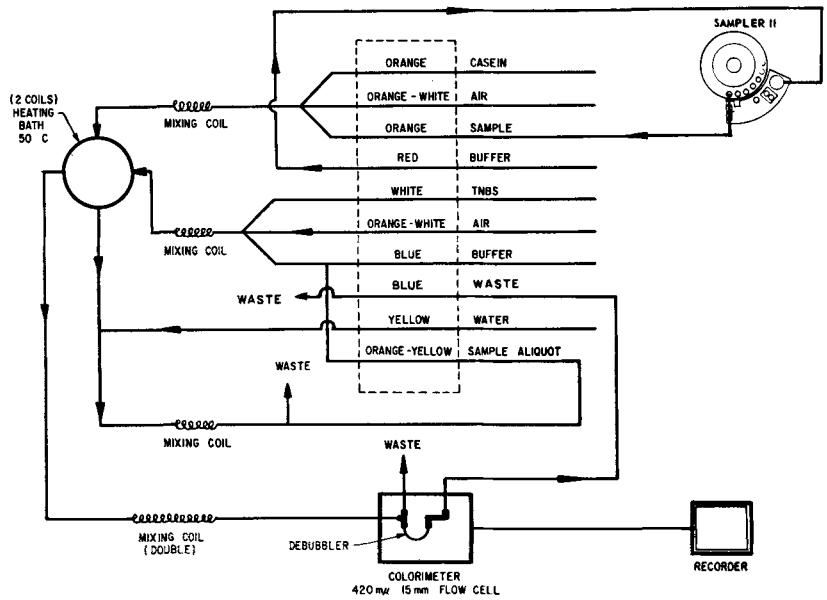


FIG. 2. Flow diagram for automated bioassay determination of enzyme in detergent.

14 min at 50 C for color development. The resulting yellow complex passes through a 15 mm flow cell, and its absorbance is recorded at 420 mμ. The absorbancies of the individual samples are then related to either milligrams of enzyme per a fixed sample volume or to weight per cent enzyme by means of a calibration curve obtained from a similar analysis using reference enzyme solutions. However, due to variations of the raw material enzyme from different suppliers, it is imperative that the calibration curve be prepared from the same enzyme used in the actual manufacturing of the formulated product.

Results and Discussion

Generally, proteolytic assays involve the hydrolysis of a protein substrate such as casein, followed by termination of the reaction and quantitative measurement of the enzymic digestion. In the flow diagram, depicted in Figure 3, a proteolytic enzyme attacks the casein substrate by hydrolyzing the peptide bonds. As a result of this cleavage, an amino group is formed. Thus, the concentration of amino groups produced determines the extent of enzymic hydrolysis.

The termination of the reaction may be accomplished by different techniques, two of which have been investigated in our laboratories (1). In one method, trichloroacetic acid (TCA) is used to lower the pH and precipitate the residual substrate and enzyme. The reaction mixture is centrifuged and the analysis is performed on the TCA soluble fraction in the supernatant liquid. The second technique involves heat inactivation, in which the enzyme is destroyed by heating the reaction mixture for 5 min at 95 C. In this method, the residual substrate is not separated out and the entire reaction mixture is analyzed. It offers a definite advantage over the TCA method in that no centrifugation is required. It should be emphasized, however, that contrary to manual proteolytic assays, the enzymic reaction in the automated system described herein is not terminated before color development. Since the samples and reference solutions are subjected to the same continuous flow operation, analytical results are not affected.

Following digestion, the amino groups formed are analyzed. Two colorimetric reagents, ninhydrin and trinitrobenzene-sulfonic acid (TNBS), were investigated for this purpose. Of the two, ninhydrin presented several problems; namely, its instability in light and air, the constant concern for peroxidation of the solvent methyl cellosolve (2), the instability and danger of using potassium cyanide to maintain ninhydrin in its reduced form (hydrindantin), and the necessity to maintain the proper solvent-water ratio to prevent any precipitation of hydrindantin (3). Furthermore, a boiling water bath was required

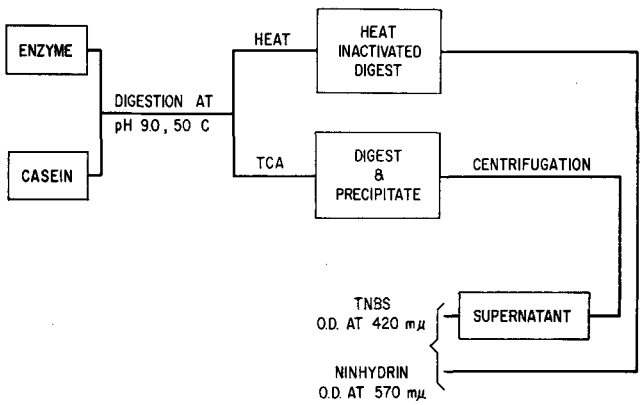


FIG. 3. Bioassay scheme.

TABLE II
Reproducibility of Results
Weight Per Cent of Enzyme Found[a]

	Same sample individual weighings	One sample individual aliquots
	0.66	0.63
	0.66	0.63
	0.63	0.63
	0.66	0.61
	0.66	0.63
	0.63	0.61
	0.66	0.61
	0.61	0.63
	0.63	0.63
	0.66	0.63
Average	0.64_6	0.62_4
Standard deviation	0.019	0.01
Coefficient of variation	2.93%	1.54%

[a] Weight based on detergent.

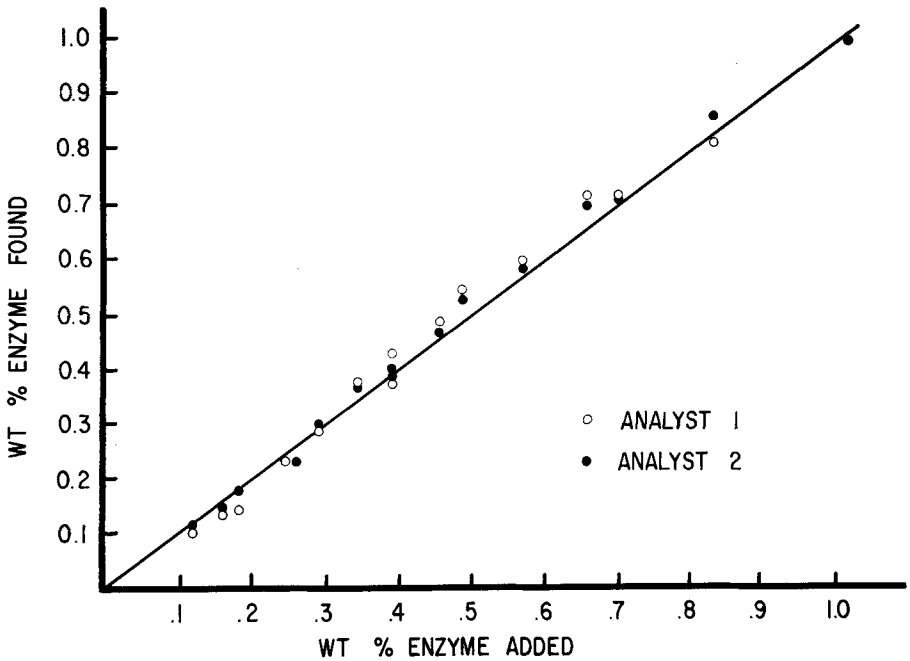
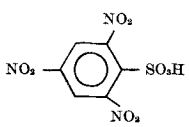


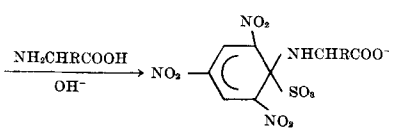
FIG. 4. Laboratory prepared standards with enzyme stock solution.

for color development. On the other hand, TNBS appeared to be workable in a relatively simple system. The difficulties just described for ninhydrin are not encountered and a boiling water bath is no longer needed for color development.

TNBS reacts with amino groups to yield the yellow Meisenheimer complex as shown below. This complex has been found to be very stable, as shown by Satake et al. (4) and verified in our own laboratories. It presented no problems in a manual operation and its adaptation to an automated system was successful.



TNBS



Meisenheimer Complex

Comparison of Methods and Colorimetric Reagents

Randomly selected detergent samples covering a wide range of enzyme concentrations were analyzed by both the manual ninhydrin and TNBS methods and the proposed automated TNBS method. A comparison of results obtained by the three procedures indicated that the automated method was reliable (Table I). An analysis of variance (ANOVA) of these data shows that at the 95% confidence level there is no significant difference of results among the three procedures [F_{calc} (2,30) = 0.163, F_{05} (2,30) = 3.32].

Accuracy and Precision of the Automated Method

The accuracy of the method was determined in a two-part experiment. First, various aliquots from an enzyme stock solution were added to detergent solutions, resulting in enzyme-detergent concentrations in

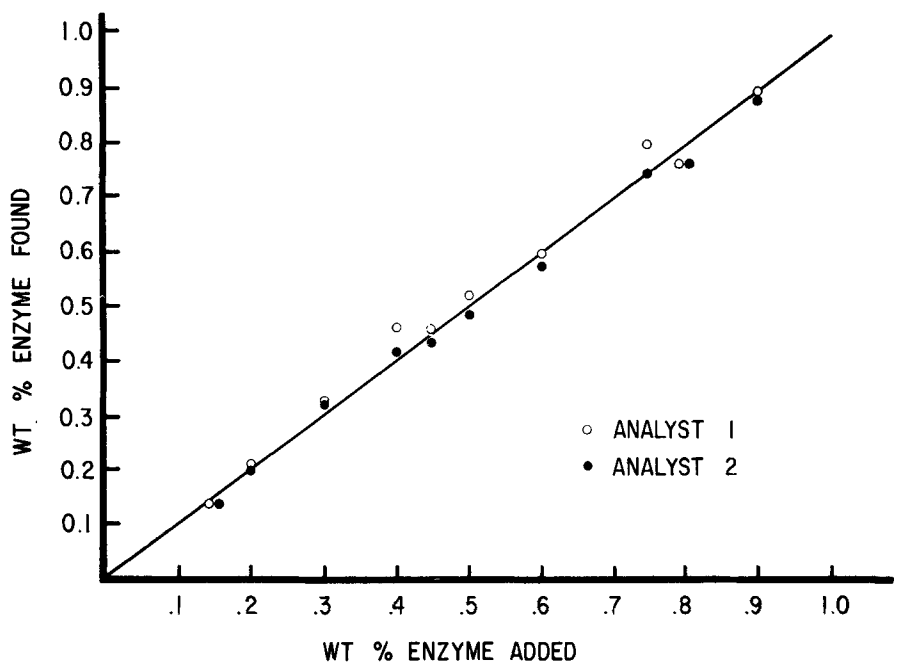


FIG. 5. Laboratory prepared standards with enzyme powder.

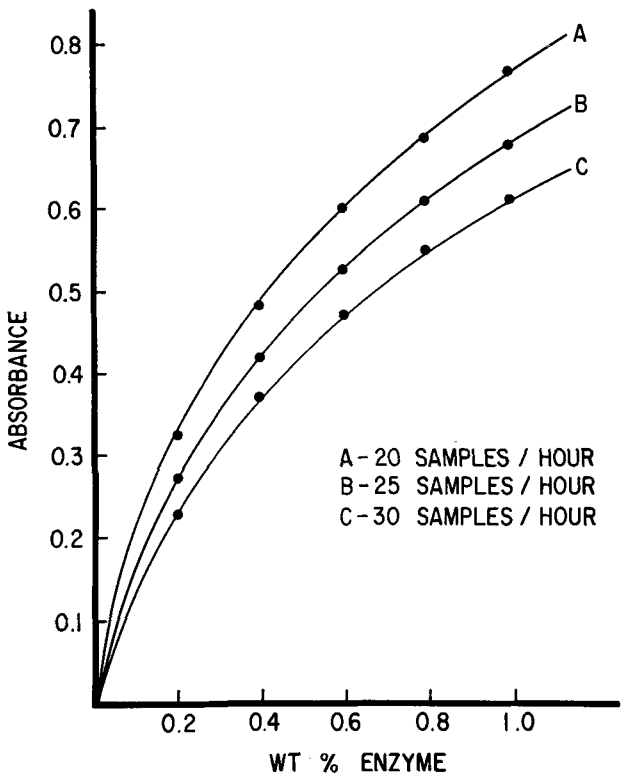


FIG. 6. Effect of rate of analysis on calibration curve.

the range of 0.12% to 1.01% w/w. Secondly, various weights of the enzyme powder were blended with appropriate amounts of dried detergent in a range of 0.15% to 0.90% enzyme-detergent. All samples were coded and analyzed independently by two analysts. Results obtained are plotted in Figures 4 and 5, where the 45-degree straight line represents 100% correlation. Statistical treatment of the data presented in both Figures shows that: (a) very high correlation coefficients of 0.997 and 0.998 for Analyst 1 and 0.995 and 0.997 for Analyst 2 were obtained on comparing their respective results with the weight per cent of enzyme added; (b) the per cent errors over the high-low ranges for the two individual analysts were 0.81% and 1.12% (Fig. 4) and 0.68% and 1.58% (Fig. 5).

The precision of the method as shown in Table II is excellent. The data shown in the left column represent analyses of 10 individual weighings from the same ground sample. A solution of one of these samples was analyzed 10 times, yielding results appearing in the right column. In the first case, variations are due both to analytical method and the sampling; in the second, the variation is due to the analytical method only.

TABLE III
Interlaboratory Reproducibility
Weight Per Cent of Enzyme Found[a]

Sample	Lab. 1	Lab. 2	Lab. 3
1	0.44	0.48	0.48
2	0.42	0.40	0.45
3	0.40	0.40	0.45
4	0.60	0.57	0.59
5	0.62	0.56	0.58
6	0.63	0.58	0.59
7	0.78	0.80	0.80
8	0.78	0.72	0.77
9	0.85	0.81	0.83

[a] Weight based on detergent.

TABLE IV
Speed of Operation
Weight Per Cent of Enzyme Found[a]

Sample	Number of samples per hour		
	20	25	30
1	0.48	0.48	0.48
2	0.48	0.46	0.48
3	0.44	0.45	0.50
4	0.50	0.50	0.47
5	0.48	0.48	0.49
6	0.53	0.53	0.55
7	0.48	0.46	0.47
8	0.51	0.51	0.53
9	0.47	0.47	0.50
10	0.48	0.48	0.47
11	0.51	0.52	0.53
Average	0.48_9	0.48_5	0.49_7
Standard deviation	0.025	0.027	0.028
Coefficient of variation	4.9%	5.3%	5.6%

[a] Weight based on detergent.

Further, analyses by three independent laboratories of nine randomly selected detergent samples showed the interlaboratory reproducibility to be very good (Table III). Statistical treatment of these data (by the paired differences method) demonstrates that, at the 95% confidence level, there is no significant difference of results between Laboratory 1 and Laboratory 2 or between Laboratory 1 and 3. However, there is a difference between Laboratory 2 and 3; all values obtained by Laboratory 3 are equal to or slightly greater than the corresponding values from Laboratory 2. The small differences observed are, in all probability, due to the nonhomogeneity of the samples.

Rate of Analyses

Since the primary stages of our investigation, speed of operation has been one of the main parameters to be considered. The automated part of the system may be operated either at 20, 25 or 30 samples per hour (actual speeds of 40, 50 or 60 samples per hour reduced to one half because of the buffer cups introduced between sample cups) with no apparent sacrifice of accuracy or resolution between peaks.

Typical calibration curves obtained at these different speeds are represented in Figure 6. As the speed of operation is increased the actual amount of sample presented to the system is decreased, resulting in lower absorbance readings. Although the color intensities are, as expected, somewhat affected on an absolute basis, the relative measurements of the individual samples calculated against their corresponding curves remain very uniform as shown by the data in Table IV. ANOVA of these data shows that, at the 95% confidence level, there is no significant difference of results obtained among the three speeds of operation.

The assay procedure herein described has been found to be free of interferences from ingredients such as tripolyphosphate and anionic surfactants at levels commonly found in detergent products. It has also been used as an investigative tool in research projects devoted to enzymes.

ACKNOWLEDGMENTS

Analytical work by C. DeRoner and J. Fucsko.

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[Received March 4, 1969]