Evaluation of Enzymes for Laundry Products[1]

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Abstract

Currently the two classes of enzymes finding favor as detergent additives are the proteases and amylases. Data are presented showing some of the desirable characteristics such enzymes must possess to be successfully employed as laundry products. Whereas it is easy to determine the activity of the enzymes with test tube assays using homogeneous substrates, translation to a practical method for laboratory evaluation in which apparent laundering ability is correlated with enzyme content has proved to be more difficult. Data are presented showing that, whereas EMPA 116 test fabric (uniformly stained with blood, milk and Japanese ink) is suitable for establishing the effectiveness of detergents containing proteases, the commonly employed EMPA 112 (stained with cocoa, milk and sugar) used for evaluating amylase-containing products is a poor choice for this purpose. This appears to be because both cocoa and milk contain proteins serving to bind the stains to the cloth. Accordingly, EMPA 112 responds to protease as well as amylase. An experimental test fabric, uniformly stained with starch and lampblack, has been prepared and has proved to respond solely to the amylase present in detergent formulations. Thus, it is now possible to evaluate the effectiveness of the amylase component in laundry products, independent of the influence of other enzyme systems that might also be present.

Introduction

Evaluation of Enzymes for Laundry Products

What has been common knowledge among the dry cleaners for over 30 years has now succeeded in engendering excitement among the "wet cleaners:" enzymes can be successfully employed to remove stains from fabrics. Why did it take the laundry product developers so long to recognize the potential of enzymes as detergent additives?

The answer is twofold. On the one hand, product development usually follows established practices, and in the United States the propensity for automating household chores seemed to preclude the use of additives requiring what was believed to be specialized handling. On the other hand, the enzymes suitable for use by the dry cleaner did not have characteristics that made them compatible with the laundry products.

Both of these drawbacks were overcome, each in its own way. Instead of the developments taking place in the United States, thereby bucking established trends, they took place in Europe, where laundry practices were more congenial with enzyme behavior. And, of course, new enzyme systems were developed which, for the first time, had characteristics which made them most suitable for use as detergent additives.

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

Experimental Procedures

Assay Method

The following basic assay procedure was employed to establish the influence of pH and temperature on the enzyme. In each case, the single parameter was varied, e.g., pH, with all other conditions as indicated. Borate buffer substituted for tris buffer for the compatibility studies.

Assay Solutions

Substrate. Dissolve 3.0 g (25 mmole) of tris (hydroxymethyl) aminomethane ultra pure (Mann Research Laboratory) in about 330 ml distilled water and adjust to pH 7.9 with diluted hydrochloric acid. Generally, 14.0 ml 1.0 N HCl will be needed.

Place 6.5 g casein (Hammersten grade, Nutritional Biochemicals Corp.) in a 600 ml beaker and add the previously prepared tris-HCl solution. Stir to disperse the casein and heat the mixture with occasional stirring for 20 min in a boiling water bath. Cool to room temperature, transfer to a 500 ml volumetric flask, and make to volume with distilled water. The solution should have pH 7.4 to 7.6 and contain 1.3% casein dissolved in 0.05 M tris buffer. It may be used for five days, but should be stored in a refrigerator when not in use.

Trichloroacetic Acid Reagent. Prepare a stock solution of 6.67 M acetic acid by dissolving 400 g glacial acetic acid (cp) to 1000 ml with distilled water. Dissolve 18.0 g trichloroacetic acid (TCA), reagent grade, and 19.0 g sodium acetate (cryst. 3 H_2O) in about 300 ml water, add 50.0 ml 6.67 M acetic acid solution, and transfer to a 1-liter volumetric flask. Make to volume with distilled water. The solution should have pH 3.5 $\pm$ 0.1. The TCA solution decomposes with time and should be discarded after one week. TCA solutions should not be pipetted by mouth.

Sodium Tripolyphosphate, 0.044 M, pH 8.5. Place 1.6 g sodium tripolyphosphate (Fisher, Cat. No. S-645) in a 150 ml beaker, dissolve in about 60 ml distilled water, and titrate with 0.1 N HCl to pH 8.5. About 7 ml of the acid will be needed. Transfer to a 100 ml volumetric flask and make to volume with distilled water. Replace the solution after five days.

Tris Buffer, pH 8.5. This is the solvent and diluent for the enzyme preparations. Place 6.0 g tris (same grade as used for the substrate solution) and 4.0 g sodium chloride, cp, in an 800 ml beaker, add about 500 ml distilled water, and titrate to pH 8.5 with 1.0 N HCl. About 14 ml of the acid will be needed. Transfer to a 1-liter volumetric flask and make to volume with distilled water.

Enzyme Solutions. Prepare all solutions and serial dilutions with tris buffer (4).

Solid preparations: Prepare a 0.5% stock solution which should be stirred (magnetically) for 30 min before serial dilutions are made.

Liquids: Weigh a 10.0 g sample into a 100 ml volumetric flask, make to volume, and keep with intermittent mixing for about 10 min before making further dilutions.

Final solutions should contain between 9 and 26

TABLE I

Compatibility of Alkaline Protease 201 With Detergent Components

Ingredient	Conc in dry mix %	Recovery after 30 min at 40 C, pH 10 (%)
None (control)	....	100
Sodium tripolyphosphate	15	92
Sodium perborate	10	95
Sodium hypochlorite	[5.7]	9
Sodium metasilicate	10	100
Tetrasodium pyrophosphate	10	95
Carboxymethylcellulose	1.5	97
Nitrilotriacetic acid	5	95
Linear alcohol ethoxylate (nonionic)	15	100
Sodium lauryl sulfate (anionic)	15	93
Alkyl dimethyl ethyl and ethyl benzyl ammonium chloride (cationic)	1	71

units per milliliter (for unit definition, see below) to give readings within the preferred range of the test.

Procedure

In a preliminary test make sure that a mixture of 3.0 ml tris buffer, pH 8.5, 3.0 ml substrate solution, 3.0 ml 0.02 N NaOH and 3.0 ml sodium tripolyphosphate solution has pH 8.5 to 8.7. When the pH has been found to be satisfactory proceed as follows:

a) Prepare a series of 25 × 150 ml tubes (one for each enzyme test and one for the substrate blank) each containing: 3.0 ml each of substrate solution, 0.02 N NaOH, and sodium tripolyphosphate solution. Pipette the three solutions either separately into the tubes or use 9.0 ml of a freshly prepared mixture. Close the tubes with No. 4 stoppers and equilibrate at 37 degrees for about 10 min.

b) At zero time add 3.0 ml enzyme solution to the tubes, except to the substrate blank which receives 3.0 ml tris buffer, pH 8.5. Mix immediately after the addition by tapping the tube for at least 30 sec.

c) Incubate the tubes for exactly 15 min at 37 C, add 10.0 ml TCA reagent, and shake thoroughly for about 30 sec. Place tubes in the 37 degree bath and shake again after 15 min. Keep another 15 min at 37 degrees and shake again vigorously. Filter through an 11 cm Whatman No. 42 paper. To obtain perfectly clear filtrates refilter the first portion of the filtrate through the same filter.

d) Enzyme blank. Incubate about 5 ml enzyme solution in a separate tube for 15 min at 37 degrees. Pipette 3.0 ml each of substrate solution, 0.02 N NaOH, and sodium tripolyphosphate into a tube, incubate 15 min at 37 degrees, and add 10.0 ml TCA solution. Mix well for at least 1 min. Add 3.0 ml of the separately incubated enzyme solution and mix well. Place the tubes in the 37 degree bath and shake again after 15 min. Keep another 15 min at 37 degrees and shake again vigorously. Filter through an 11 cm Whatman No. 42 paper. Refilter the first portion of the filtrate through the same filter.

e) Read the optical densities of the filtrates at 277 mμ using a 10 mm cell in a suitable spectrophotometer set at zero with the filtrate of the substrate blank. Correct the readings of the enzyme test by subtracting the value of the enzyme blank containing the same amount of enzyme. When testing different amounts of the same enzyme sample, run the enzyme blank only with the highest enzyme concentration and calculate the corrections for lower ones by interpolation.

Definition of Units

One unit of activity is that amount of enzyme which produces in 1 min under conditions of the test a hydrolyzate whose absorbance at 277 mμ is the same as the one of a tyrosine solution containing 1.50 μg tyrosine per milliliter.

Calculation of Units

The number of units in the digestion mixture is:

$$\frac{A_{277} \text{ of hydrolyzate}}{A_{277} \text{ of 1.5 } \mu\text{g ty/ml}} \times \frac{\text{volume (ml)}}{\text{time (min)}} =$$

$$\frac{A_{277} \text{ of hydrolyzate}}{0.0108} \times \frac{22}{15} =$$

$$A_{277} \text{ of hydrolyzate} \times 136$$

The units found with this method are designated "PCA units." The number of PCA units per gram of a preparation is called the PCA of the preparation. Thus:

$$\text{PCA} = \frac{A_{277} \text{ of hydrolyzate} \times 136}{\text{gram of enzyme prep. in test}}$$

Preparation of Starch Cloth

Bleached linen is padded with a mixture containing approximately 1.5% lampblack and 3% gelatinized starch. A suitable quantity of this test fabric can be prepared in the laboratory in the following manner:

Thoroughly disperse 25 g of lampblack in 100 g of methanol, and dilute with 500 ml of water. Prepare a second mixture containing 50 g of acorn starch and 950 ml of distilled water. Heat this slowly to 90 C with rapid mixing, and cool to 40 C. Add the lampblack suspension and continue stirring for 30 min.

This mixture is suitable for padding 10–12 yards of the bleached linen, 6 in. wide. This is done by immersing the cloth in an appropriate container of the mixture, passing the cloth between rubber rollers, and repeating the process a second time. The padded cloth is then dried in an oven for 30 min at 88 C. It is advisable to allow the cloth to age for at least 24 hr at room temperature before use. This cloth has been designated Experimental Starch Cloth 2B.

Results

Enzyme Characteristics in Vitro

Early development work in Europe established that the most dramatic laundering results were obtained with proteolytic enzymes isolated from varieties of *Bacillus subtilis*. When the microorganism is grown under certain prescribed conditions, a type of protease is produced that has several unique characteristics:

1. An extremely high pH optimum which in many instances is in excess of pH 10. A typical pH-activity curve can be seen in Figure 1; the optimum of Alkaline Protease 201 from Wallerstein Company is 10.9, using casein as the substrate and 37 C as the temperature of incubation.

2. A suitably high temperature optimum. This can be seen in Figure 2, which shows the optimum to occur at approximately 60 C. An interesting phenomenon occurs when one attempts to combine these characteristics, i.e., optimum pH with optimum temperature. As can be seen in Figure 3, when the pH-activity curve is repeated at 61 C there is a shift

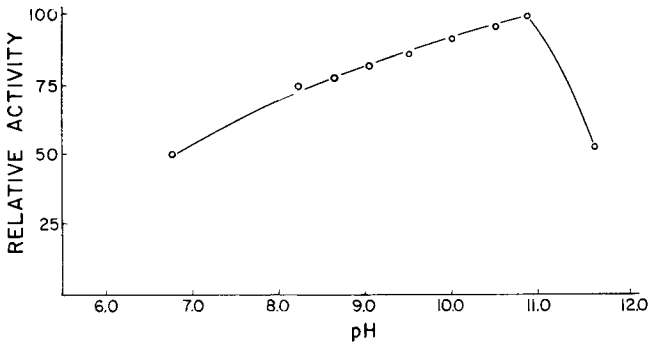

FIG. 1. Activity as a function of pH, Alkaline Protease 201.

towards the acid side resulting in a new optimum at pH 9.6. This characteristic is well known to enzyme chemists and is the result of a competition between activity and destruction of the enzyme at the higher temperature and pH. As the temperature and pH are raised, a point is reached where the destructive influence of both of these on the enzyme protein and activity is manifested by the shift of the curve away from the harsh environmental conditions.

An additional point of interest established by these data is the increase in the absolute maximum activity by a factor of 3 when going from 37 to 61 C. In other words, even though the pH optimum shifts and the relative activity is lower at pH 10.9 than at 9.6 at the higher temperature, the absolute activity remains greater than that attained at the lower temperature.

3. The protease also exhibits excellent pH-stability characteristics, as can be seen from the broad plateau shown in Figure 4. The enzyme was incubated at the indicated pH, in solution, for 19 hr at 35 C.

4. Since so many enzymes are inhibited by sodium tripolyphosphate (STP), an important characteristic of an enzyme suitable as a detergent additive must be compatibility with this ubiquitous ingredient. Any meaningful assay method for the activity of detergent enzymes should incorporate STP as a component of the incubation mixture. This is done to make certain that the activity being measured is exclusive of any neutral protease which may be present as a contaminant of the alkaline protease, a relatively simple task because of the inhibition of the neutral enzyme by the STP.

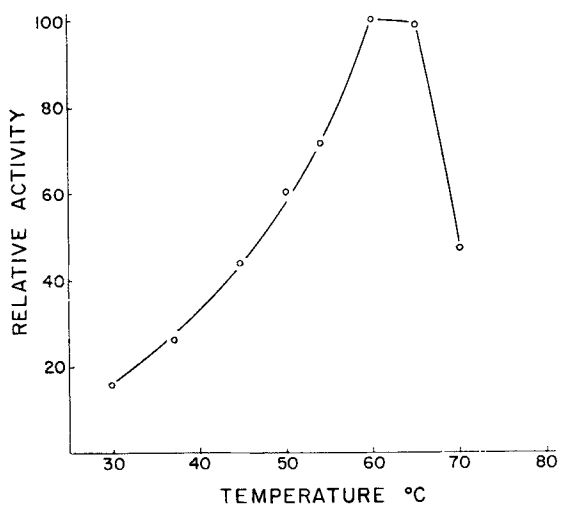

FIG. 2. Activity as a function of temperature, Alkaline Protease 201.

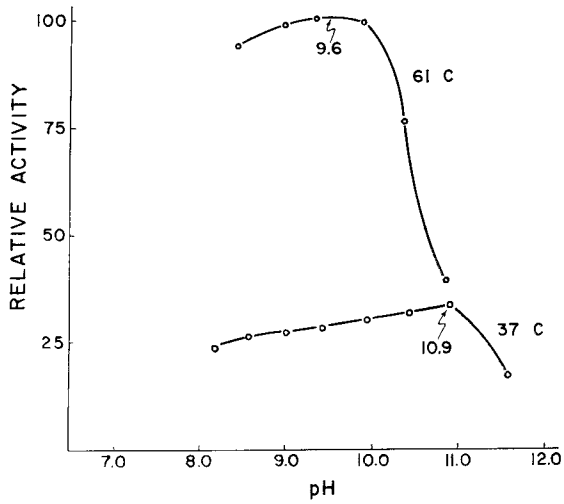

FIG. 3. pH-Activity as a function of temperature, Alkaline Protease 201.

Table I shows the compatibility of Alkaline Protease 201 with some typical components found in today's laundry products. Dry mixes were made with the enzyme at a level of 1% and the test compound at the level indicated in the Table. The mix was added to borate-buffered water at pH 10, at a level corresponding to approximately one cup per 15 gal of water. This was allowed to incubate at 40 C for 30 min, after which an aliquot was removed for assay of the enzyme. Under these conditions, compatibility with sodium tripolyphosphate, sodium perborate, sodium metasilicate, tetrasodium pyrophosphate (TSPP), carboxymethyl cellulose (CMC), nitrilotriacetic acid (NTA), a linear alcohol ethoxylate (nonionic surfactant) and sodium lauryl sulfate (anionic surfactant) is excellent. With a quaternary cationic base, such as a mixture of alkyl dimethyl ethyl and ethyl benzyl ammonium chloride, compatibility can be classified as fair to good, whereas it is very poor with the chlorine bleach.

Enzyme Characteristics in Vivo, Practical Tests

The above data were obtained by measuring the activity of the enzyme on an isolated substrate, in this case casein. Whereas such information is extremely useful, it is obviously dangerous to extrapolate in vitro information of this type to the actual laundry operation.

To evaluate these products under more practical conditions, but at the same time with some degree of standardization and reproducibility, standard test fabrics have been employed, and a soaking or full-cycle operation has been approximated in laboratory equipment.

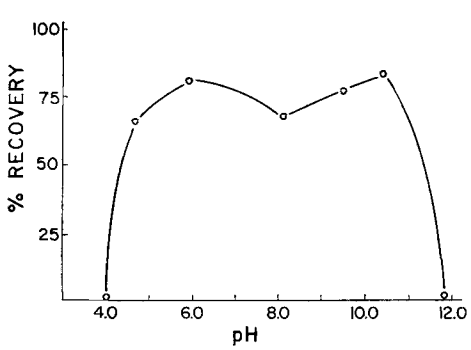

FIG. 4. Stability as a function of pH, Alkaline Protease 201.

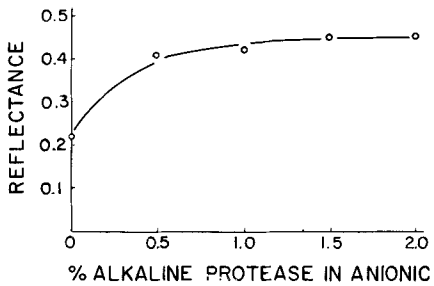

FIG. 5. Effect of enzyme level on laundering of EMPA 116.

The selection of the washing equipment will not be discussed here, though the standard Terg-O-Tometer or Launder-Ometer is perfectly satisfactory.

EMPA test fabric (Test Fabrics Inc., N.Y.) has been successfully used as soiled cloth, especially for the proteolytic enzymes. For example, EMPA 116 is uniformly stained with blood, milk and Japanese ink, and can be used effectively to establish the level of enzyme required, efficacy of the total product, etc. Figure 5 shows data obtained with EMPA 116 to determine the optimum enzyme concentration with an anionic formulation. A Launder-Ometer was used at 40 C, 20 min wash cycle, and reflectance readings taken with a Gardiner reflectometer with a blue filter. It can be seen that whereas maximum reflectance is exhibited in this test system with an enzyme level of 1.5%, the percentage difference between maximum reflectance and that obtained with 0.5% enzyme is less than 10%. However, it is possible to visually distinguish between test fabric laundered with 0.5% enzyme and 1.0% or 1.5% enzyme. In other words, the eye appears to be more sensitive as a titration mechanism than the automatic reflectometer when using EMPA 116 as the test fabric.

Typical of one of the dangers inherent in the extrapolation of in vitro data is the finding summarized in Figure 6. A second temperature-activity curve was obtained using the Launder-Ometer and EMPA 116 as the test vehicle. The fabric was washed for 15 min at the indicated temperatures, with an anionic basic formulation or with the base plus Alkaline Protease 201 at a level of 0.8%.

It can be seen that the detergent plus enzyme has a temperature optimum between 40 C and 50 C, rather than above 60 C as previously described for the in vitro test system (see Fig. 2).

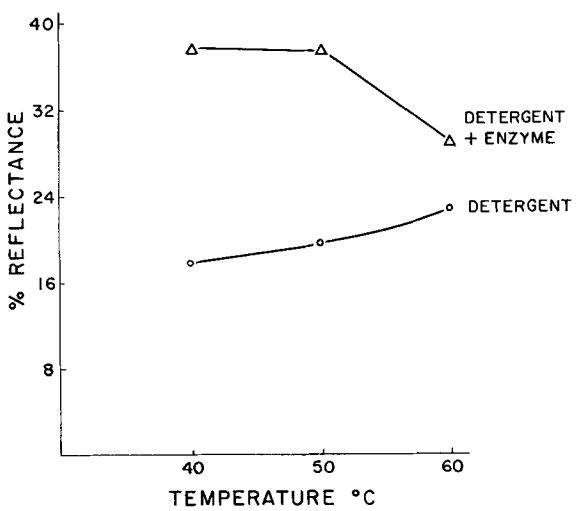

FIG. 6. Activity as a function of temperature, EMPA 116, Alkaline Protease 201.

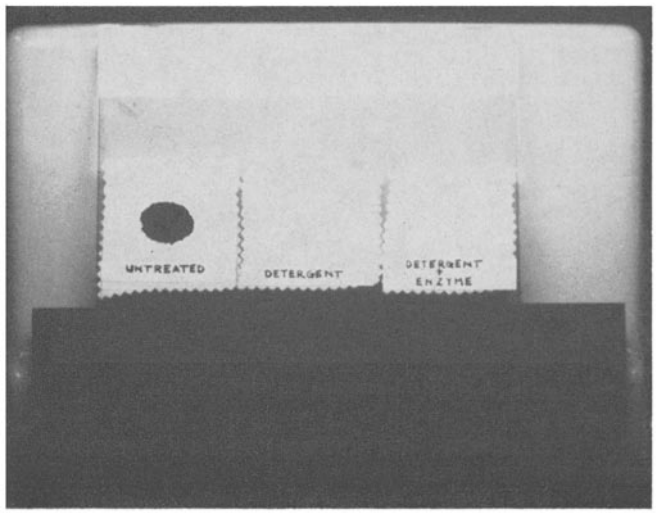

FIG. 7. Effect of enzyme on human blood stain.

In addition, it can be seen that the detergent base alone is more efficient at the higher temperature, though the enzyme-containing product is more effective at all temperatures tested.

Since it is hard to visualize the cleaning power of the enzymes when using the artificially stained fabrics, a laundry test was performed using cloth spotted with human blood as the test vehicle.

Blood was applied to white Indianhead cotton, the cloth was allowed to dry at room temperature and then aged for one week. The laundry tests were made at 50 C, 15 min wash cycle. Figure 7 shows the effect of the anionic base alone and the detergent plus Alkaline Protease 201. The effectiveness of the protease is clearly seen.

Today there is increasing interest in what can be described as the second-generation enzymes, that is, the combination products containing an amylase as well as the protease. Varieties of *B. subtilis* also secrete an amylase that is functional at the high pH of laundry products and reasonably compatible with most of the components normally found in these products.

In vitro assays of amylase are well known and will not be dwelt on here. However, the test fabric widely used for evaluating amylase as an in vivo counterpart to EMPA 116 is EMPA 112. This cloth is uniformly stained with cocoa, milk and sugar, and has been quite universally employed for this purpose. A typical titration curve under conditions as previously described for the protease with EMPA 116 can be seen in Figure 8. In this case the combination product, Alkaline Protease HA from Wallerstein

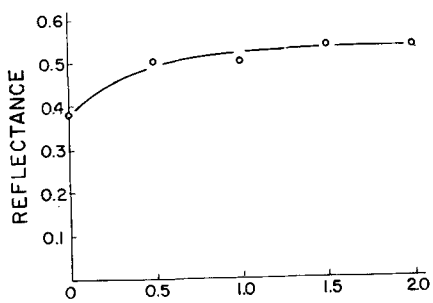

FIG. 8. Effect of enzyme level on laundering of EMPA 112.

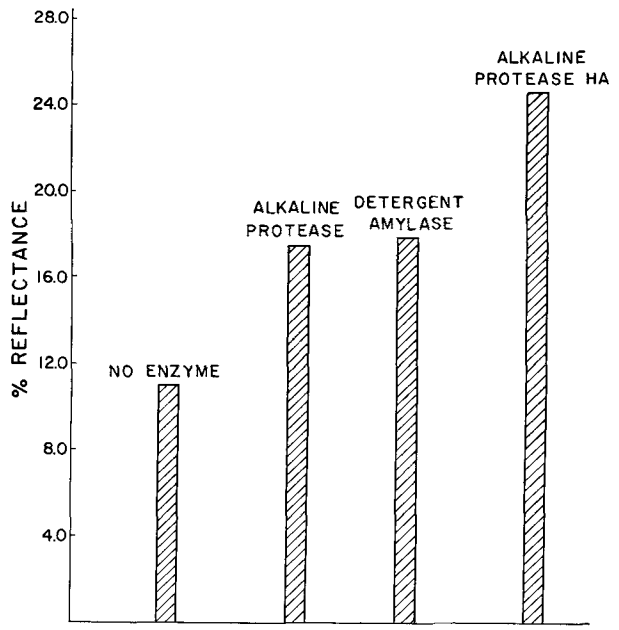

FIG. 9. Influence of protease and amylase on EMPA 112.

Company, was employed. This consists of both the protease and the amylase, each at levels previously established to provide maximum laundering efficiency when used in combination at a final concentration in the laundry product of between 0.5% and 1.25%. While the shape of the curve is similar to that obtained with the protease on EMPA 116, it can be seen that the anionic base, in the absence of enzyme, is capable of quite effective laundering of this test fabric. In other words, this fabric yields a high reagent blank. In addition, visual observations on EMPA 112 are extremely difficult to make, since the tan background does not provide a suitable base for making fine distinctions.

During the course of evaluating a number of enzyme preparations on these test fabrics, it was established that the protease was the only enzyme that increased the effectiveness of the various laundry detergents with EMPA 116. However, it soon became apparent that EMPA 112 was responding not only to amylase but to protease as well. This is shown in Figure 9. It can be seen that the isolated amylase

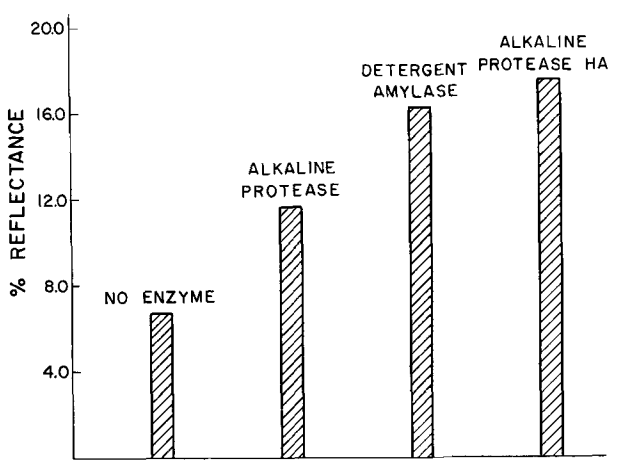

FIG. 10. Influence of protease and amylase on Experimental Starch Cloth 1B.

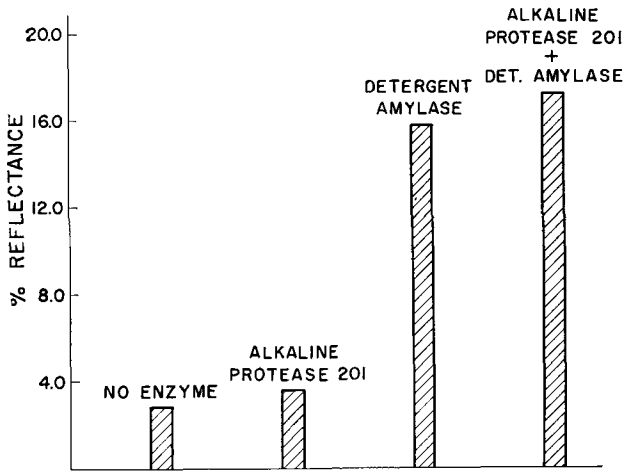

FIG. 11. Influence of protease and amylase on Experimental Starch Cloth 2B.

really does not do much more than isolated protease, but that the two in combination are much more effective than each alone.

This apparent synergism is easily reconciled if we recall the stains employed for EMPA 112. Two of the three stains (cocoa and milk) contain proteins which undoubtedly serve to bind the stain to the cloth. It is, therefore, not at all surprising to learn that EMPA 112 is a poor test fabric for the evaluation of the efficiency of amylases, and should not be considered as an appropriate test vehicle for anything but combination products.

We have attempted to prepare a test fabric that might be more suitable for use as an indicator for amylase. In one of the early trials a bleached desized cotton was uniformly stained with gelatinized starch and lampblack, blended with suitable dispersing agents, and designated Experimental Starch Cloth 1B. The dried fabric was aged and evaluated as a test cloth for amylase in detergent formulations, using the same techniques as described for EMPA 112.

Figure 10 shows that whereas this experimental fabric does respond more specificially to the amylase component in the enzyme mixture, there is also a distinct response to the protease component, despite the absence of protein in the stain. In addition, while better than EMPA 112, it also displays the high reagent blank and is quite effectively laundered by the base detergent in the absence of enzyme.

Our most recent experimental test fabric is also stained with gelatinized starch and lampblack, but applied in a slightly different manner, as described in the Experimental Procedures section. Figure 11 shows the influence of protease and amylase on this cloth. It can be seen that: The response to the basic detergent is minimal; there is virtually no response to the protease; there is an excellent response to the amylase; and the response to the mixture is due solely to the amylase.

Thus, it would appear that this test cloth is well suited for evaluating amylases in laundry products.

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