Enzymes for Low Temperature Washing[1]

M.H. NIELSEN, S.J. JEPSEN and **H. OUTTRUP,** Novo Research Institute, Novo Allé, 2880 Bagsvaerd, Denmark

ABSTRACT

The increasing use of synthetic fibers which cannot tolerate temperatures above 50–60 C has changed the washing habits during the past 5–10 years toward the use of lower washing temperatures. Furthermore, the energy crisis has focused interest on washing at ambient temperatures for the purpose of saving energy. In order to compensate for the lower washing efficiency at decreased temperatures, enzyme producers have devoted much R&D capacity to screen for new proteolytic enzymes which are more suitable for washing at lower temperatures. The result of a screening program carried out for several years, a new detergent protease with interesting characteristics, is discussed.

INTRODUCTION

The washing methods used in different parts of the world can be roughly divided into three main segments: the hot-wash area, Europe; the warm-wash area, USA; and finally, the cold-wash area, comprising Japan and Southeast Asia. Detergent proteases are used to a large extent in the hot-wash area due to their excellent effect under the washing conditions currently used. The situation is different in the warm wash area as the detergent proteases developed until now have a less pronounced effect at lower temperature washing conditions, which is illustrated by the relatively small use of enzymes in detergent compositions. In the cold-wash area, where washing temperatures of 10–25 C are predominant, the addition of enzymes has only a marginal effect and they are only used in special formulations in which a synergistic effect between the enzyme and special detergent ingredients improve the efficiency.

The traditional pattern of washing habits outlined is changing, partly due to the rising energy costs and partly to the increasing use of synthetic fibers which can only tolerate moderate temperatures. Therefore, a tendency is observed all over the Western world toward the use of lower washing temperatures with a decreased washing efficiency as the consequence.

An increase in detergent efficacy can be obtained by the incorporation of enzymes (1), but as the catalytic effect decreases rapidly with the temperature—ca. twice per 10 C—adequate concentrations in low temperature detergents are normally economically prohibitive. With this background, it is quite natural that the enzyme manufacturing industry has allocated considerable R&D resources in order to find new proteases with a better detergent efficacy at low temperatures than is the case with the present ones which are based on the enzyme, Subtilisin Carlsberg, e.g., Alcalase.

The purpose of this paper is to describe a number of washing experiments with a newly developed alkaline protease produced by a strain of the *Bacillus subtilis* group. The enzyme, which is still in the developmental phase, has a better detergent efficacy at low temperatures than Alcalase. In all the experiments, Alcalase has been used as a standard detergent enzyme. The new alkaline protease has been given the temporary designation SP 226.

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MATERIALS AND METHODS

Enzymes

Enzymes used were: alkaline protease, SP 226, batch PKF2: activity, 1.5 Anson units/g; Alcalase M, activity, 1.5 Anson units/g; crystalline Subtilisin Carlsberg, activity, 27 Anson units/g; crystalline Subtilisin Novo, activity, 27 Anson units/g.

Detergents and Other Chemicals

Commercially available U.S. and European heavy-duty detergents were used. Ovomucoid inhibitor 11-0 was from Sigma.

Test Materials

EMPA 116: cotton soiled with blood, milk and carbon black.

Denatured egg/polyester cotton swatches: whole eggs were homogenized with 0.5% drawing ink and 0.1% ethoxylated nonyl phenol. Polyester/cotton swatches were immersed in the soiling mixture and, immediately afterward, squeezed between rubber rollers. After drying overnight at ambient temperature, the egg protein was denatured by soaking the swatches for 10 min in tap water at 60 C, rinsing in running tap water, squeezing between rubber rollers and, finally, drying overnight at ambient temperature.

Native egg/polyester-cotton swatches: the procedure is identical to that described for denatured egg/polyester-cotton, except that the denaturation procedure is omitted.

Denatured egg/cotton swatches: the procedure is similar to that used for denatured egg/polyester-cotton.

Native egg/cotton: the procedure is identical to that used for native egg/polyester-cotton.

Spinach/cotton: cotton swatches were immersed in spinach juice, squeezed between rubber rollers and dried at ambient temperature. The impregnation with spinach juice was repeated. In order to denature the protein, the dry swatches were then soaked in tap water at 70 C for 10 min, rinsed in running tap water, squeezed between rubber rollers and finally dried overnight at ambient temperature.

Blood/acrylic swatches: polyacrylic fabric was immersed in citrated ox blood, immediately thereafter squeezed between rubber rollers and dried overnight at ambient temperature. The swatches were artificially aged by soaking for 8 min in tap water at 60 C, followed by rinsing, squeezing and drying at ambient temperature overnight.

The dimension of all swatches was 5 x 9 cm.

Washing Procedures

The details of the procedures used for washing are given in Tables I and II. After washing, the test swatches were rinsed in running tap water and ironed. Whiteness was measured with an Elrepho reflection photometer with filter R46 against a MgO-standard.

For every experiment, a control without enzyme was done and the difference (ΔR) between the remission values

TABLE I

U.S. Laboratory Washing Procedures

	Cold water wash Terg-O-Tometer	Warm water wash Terg-O-Tometer
Detergent conc. (g/ℓ)	1.5	1.5
Washing time (min)	30	15
Washing temperature (C)	20	50
Volume of washing solution (ml)	1,200	1,200
Number of swatches/pot	12	12

TABLE II

European Laboratory Washing Procedures

	Warm water wash Launder-O-Meter	Hot water wash Launder-O-Meter
Detergent conc. (g/ℓ)	5	5
Washing time (min)	app. 40	app. 40
Washing temperature (C)	15→60	15→90
Volume of washing solution(ml)	300	300
Number of swatches/pot	3	3

measured in the experiment and in the control was used as a measure of the effect. The experiments in the Terg-O-Tometer were done in duplicate, and in the Launder-O-Meter tests, in triplicate.

Determination of Proteolytic Activity

The activity toward hemoglobin was determined according to the Anson method (2). The activity toward casein was determined as follows: 10 ml of a 1% solution of Hammarsten casein in 0.2 M phosphate buffer, pH 7.5, + 2 ml deionized water were preincubated for 10 min at 30 C. Two ml of a buffered enzyme solution was added and the reaction mixture incubated for 30 min. The reaction was stopped by the addition of 5 ml of a 2% trichloroacetic acid solution. The mixture was kept for 10 min at 30 C and then filtrated through Whatman No. 42. The optical density of the clear filtrates was measured at 276 nm. A blank was run following the same procedure, except that the trichloroacetic acid solution was added before the enzyme solution. As the standard curve is linear, the difference in optical density between the sample and the blank was used directly as a measure for the proteolytic activity.

RESULTS AND DISCUSSION

Characteristics of the Alkaline Protease SP 226

SDS-gel electrophoresis of the enzyme preparation, batch PKF 2, with an activity of 1.5 Anson units/g, shows that more than 70% of the protein is of enzymatic origin. By comparison with marker proteins, the MW is estimated at ca. 28,000 Dalton and the specific activity at ca. 45 Anson units/g. These values are very close to corresponding ones for Subtilisin Carlsberg (3). However, SP 226 is immunologically different from both Subtilisin Carlsberg and Subtilisin Novo, which is shown by the absence of precipitates toward antisera against the two enzymes in an Ouchterlony immunodiffusion test (4). The enzyme is almost completely inhibited by phenyl methane sulfonyl fluoride, PMSF, and must, therefore, be of the serine type.

Of special interest in connection with the use of SP 226 as a detergent enzyme is the inhibition by stain components. In a series of experiments, it has been shown that the enzyme is only partly inhibited by egg white and isolated ovomucoid inhibitor (Sigma Ovomucoid Inhibitor 11-0). In Table III, the result of an experiment is shown in which the inhibition of SP 226 is compared to that of crystalline Subtilisin Carlsberg and crystalline Subtilisin Novo. It appears that SP 226 is only 40-50% inhibited whereas Subtilisin Carlsberg is inhibited almost 100%. Subtilisin Novo is less inhibited than the Carlsberg enzyme but considerably more than SP 226.

In Figures 1 and 2, the dependence of activity upon pH is shown for SP 226 and Alcalase, the activity measurements being made according to the Anson hemoglobin method and the casein method, respectively. For both enzymes, the pH-optimum is pH 8.5 when using hemoglobin as a substrate, and pH 10.5 with casein as a substrate. SP 226 has its temperature optimum at ca. 55 C, which is 5-10 C lower than that of Alcalase (Fig. 3).

TABLE III

Inhibition of SP-226, Subtilisin Carlsberg and Subtilisin Novo by Egg White and Ovomucoid Inhibitor

	Residual activity in % after incubation for 30 min	
	Egg white diluted in buffer	Ovomucoid inhibitor
SP 226	54	60
Subtilisin Carlsberg	2	4
Subtilisin Novo	18	22

Method of analysis: hemoglobin method
pH: 7.5

From Figure 4, it appears that the enzyme has rather good stability, up to 45 C, whereas at 55 C, the activity decreases rapidly with time. At 65 C, the enzyme is immediately inactivated. In this context, it shall be mentioned that the decay curve for Alcalase at 65 C (Fig. 5) is nearly equivalent to the corresponding curve for SP 226 at 55 C. The characteristics of the new enzyme indicate that it will be particularly well suited for washing at temperatures below 55 C, and in cases where the protein stains have native egg as a component. The validity of this hypothesis will be discussed in the following text, in which a number of washing experiments are described.

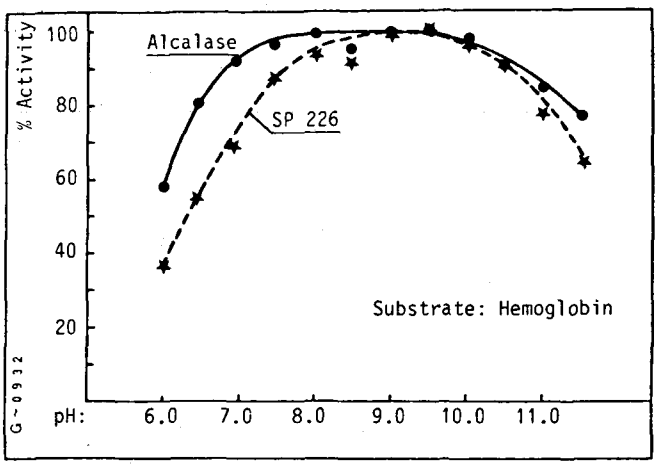

FIG. 1. Effect of pH on the activities of SP-226 and Alcalase. Substrate: hemoglobin.

Washing Experiments

Cold water washing. The concept behind the microbial screening program which has led to the new alkaline protease was that, at low washing temperatures, the proteins in the stains are not denatured and, therefore, the search should be directed toward enzymes with a pronounced effect upon native proteins. In the screening phase, a U.S. cold water wash in a Launder-O-Meter was used and hundreds of filtered culture broths were tested using Alcalase as a control. In Figure 6, the results from one of these experiments are shown. A relatively high enzyme concentration, 0.06 Anson units/ℓ, was used in order to secure maximal detergency effect. It appears that SP 226 has a better effect than Alcalase on all test swatches, and especially notable is the effect on the cotton swatch stained with native egg. The reason for this outstanding effect must be that SP 226 is only partly inhibited by the ovomucoid inhibitor whereas Alcalase is inhibited nearly 100%.

This experiment has been repeated using a more realistic enzyme concentration, 0.03 Anson units/ℓ, and the results illustrated in Figure 7 confirm the first experiment. There is still an overall better effect of SP 226 and the special effect on native egg/cotton is again observed.

After these preliminary experiments, the production of SP 226 was transferred to pilot plant scale and in the following test, a number of experiments with a preparation with an activity of 1.5 Anson units/g, which is equivalent to the activity of standard Alcalase, will be discussed. A top loading agitator-type machine, the Terg-O-Tometer, was used and the temperature kept at 20 C for 30 min (Fig. 8). The effect of SP 226 was again better than that of Alcalase with regard to all swatches, but a negative effect was observed with the native egg/cotton swatches, a phenomenon possibly caused by redeposition of the protein soiling. For both enzymes, a remarkably good effect on blood/acrylic swatches was observed.

The reason for the negative enzyme effect may be the relatively low ratio between detergent solution and test swatches, which in this case was 100:1. Also, the relatively weak mechanical treatment in the Terg-O-Tometer may have been a contributing factor and in order to eliminate these negative factors in laboratory experiments, a similar experiment was performed in a full-size, top-loading machine. To ensure realistic washing conditions, a total of 40 g of test swatches were sewn onto pure cotton fabric and, furthermore, 3 kg of clean cotton fabric was added to the washing load. Using these experimental conditions, the negative enzyme effect was reversed and both SP 226 and Alcalase showed an extraordinarily high detergency effect upon the test swatch in question (Fig. 9). With the other test swatches, SP 226 had a better or equivalent effect to that of Alcalase.

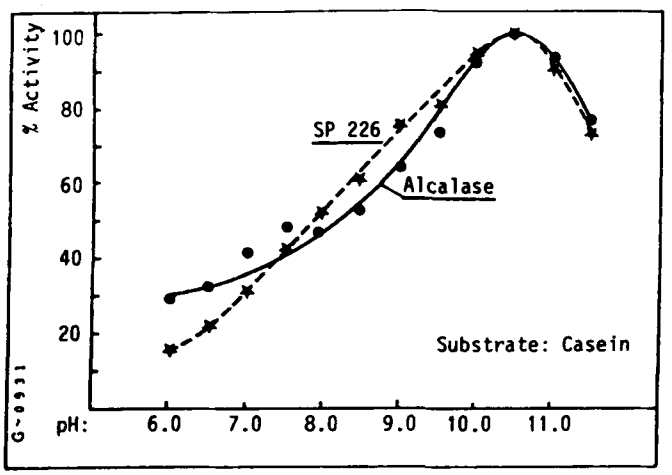

FIG. 2. Effect of pH on the activities of SP-226 and Alcalase. Substrate: casein.

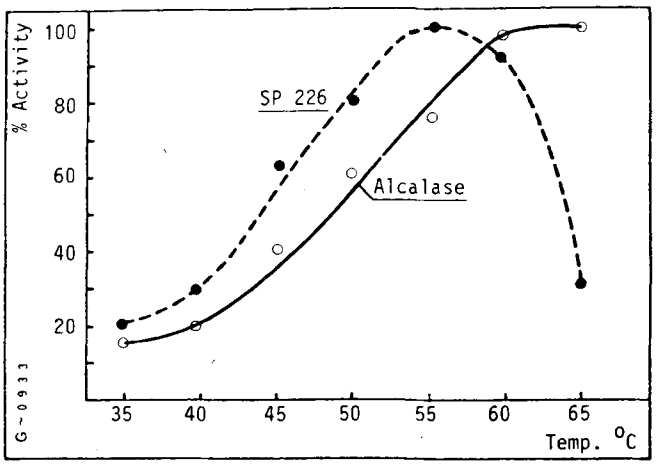

FIG. 3. Effect of temperature on the activities of SP-226 and Alcalase. Substrate: casein.

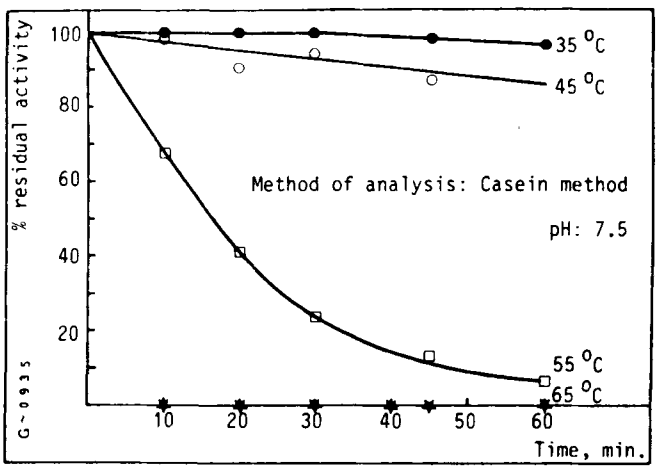

FIG. 4. Effect of temperature on the stability of SP-226. Method of analysis: casein method. pH: 7.5.

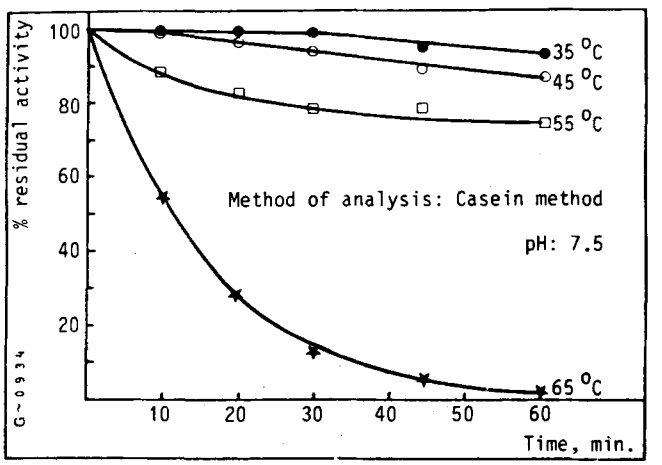

FIG. 5. Effect of temperature on the stability of Alcalase. Method of analysis: casein method. pH: 7.5.

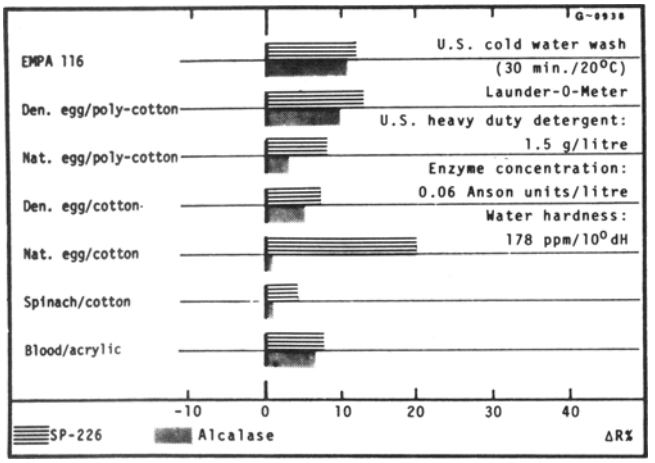

FIG. 6. U.S. cold water wash (30 min/20 C), using Launder-O-Meter. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.06 Anson units/ℓ.
Water hardness: 178 ppm/10° dH.

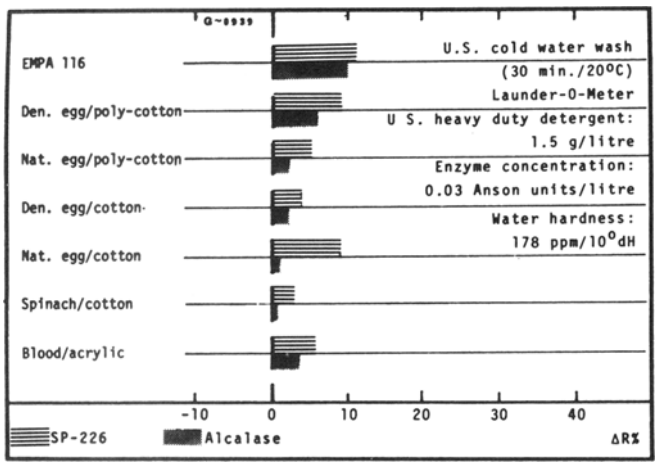

FIG. 7. U.S. cold water wash (30 min/20 C) using Launder-O-Meter. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.03 Anson units/ℓ.
Water hardness: 178 ppm/10° dH.

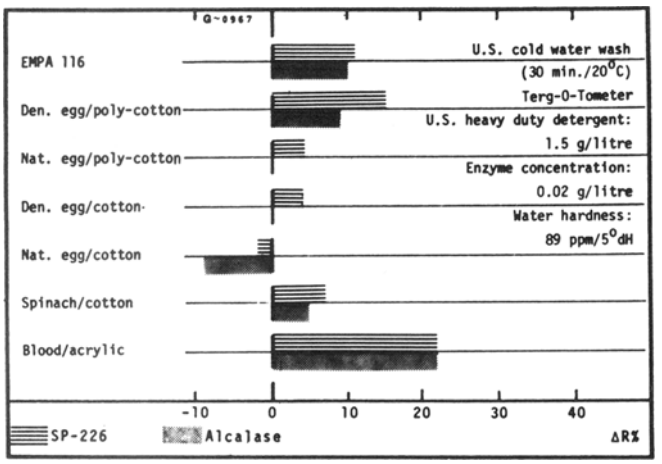

FIG. 8. U.S. cold water wash (30 min/20 C) using Terg-O-Tometer. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.02 g/ℓ.
Water hardness: 89 ppm/5° dH.

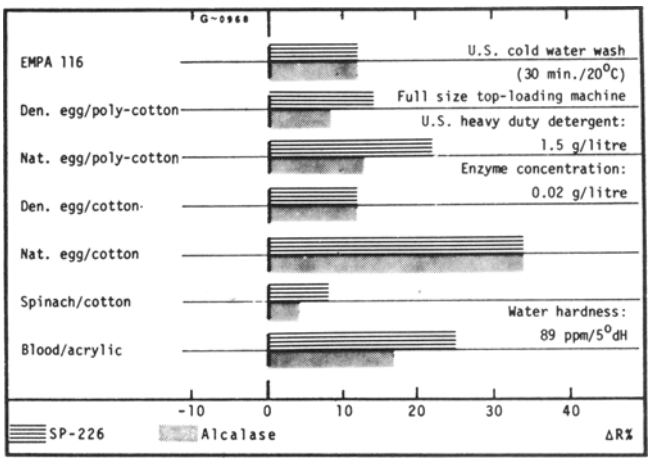

FIG. 9. U.S. cold water wash (30 min/20 C), using full-size, top-loading machine. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.02 g/ℓ.
Water hardness: 89 ppm/5° dH.

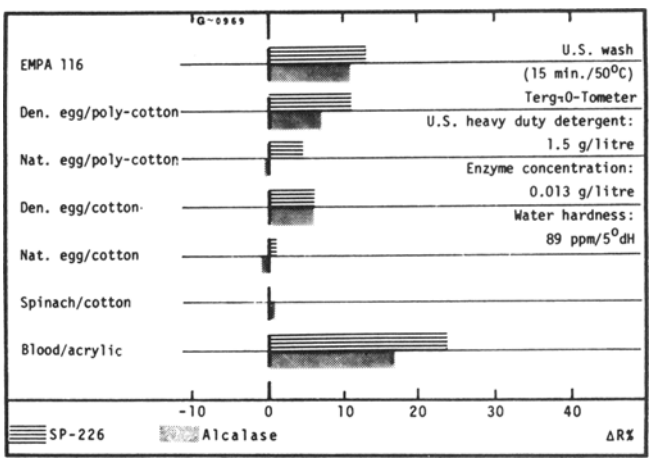

FIG. 10. U.S. wash (15 min/50 C) using Terg-O-Tometer. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.013 g/ℓ.
Water hardness: 89 ppm/5° dH.

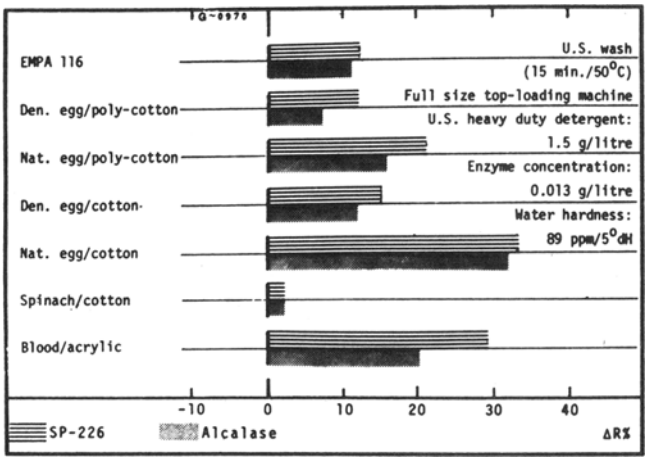

FIG. 11. U.S. wash (15 min/50 C) using full-size, top-loading machine. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.013 g/ℓ.
Water hardness: 89 ppm/5° dH.

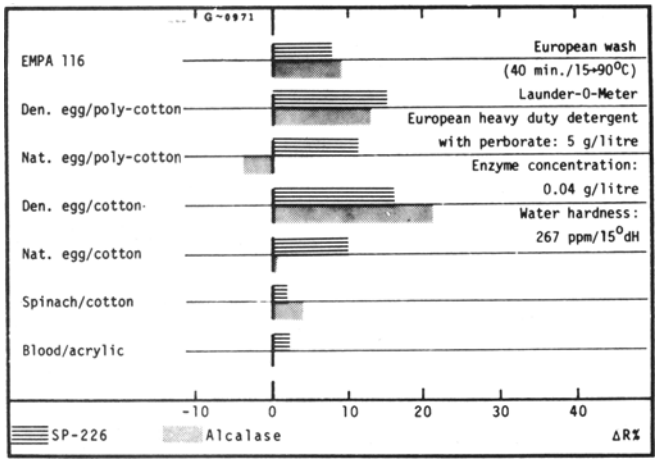

FIG. 12. European wash (40 min/15→90 C) using Launder-O-Meter. 5 g European heavy-duty detergent with perborate/ℓ. Enzyme concentration: 0.04 g/ℓ. Water hardness: 267 ppm/15°dH.

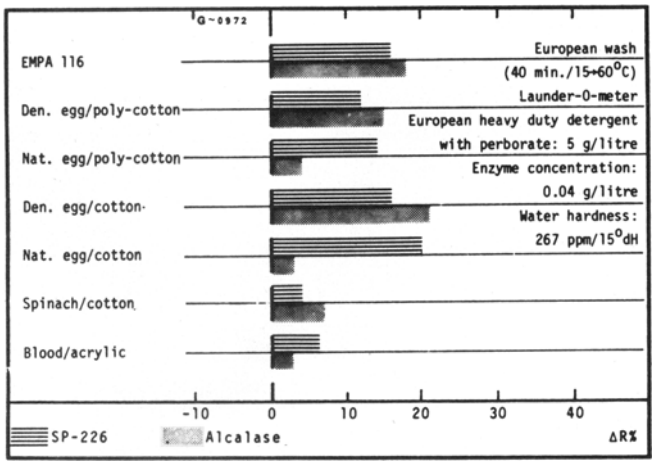

FIG. 13. European wash (40 min/15→60 C) using Launder-O-Meter. 5 g European heavy-duty detergent with perborate/ℓ. Enzyme concentration: 0.04 g/ℓ. Water hardness: 267 ppm/15°dH.

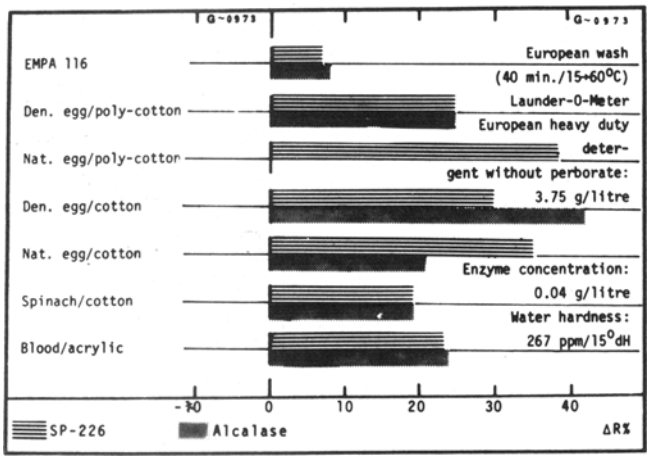

FIG. 14. European wash (40 min/15→60 C) using Launder-O-Meter. 3.75 g European heavy-duty detergent without perborate/ℓ. Enzyme concentration: 0.04 g/ℓ. Water hardness: 267 ppm/15°dH.

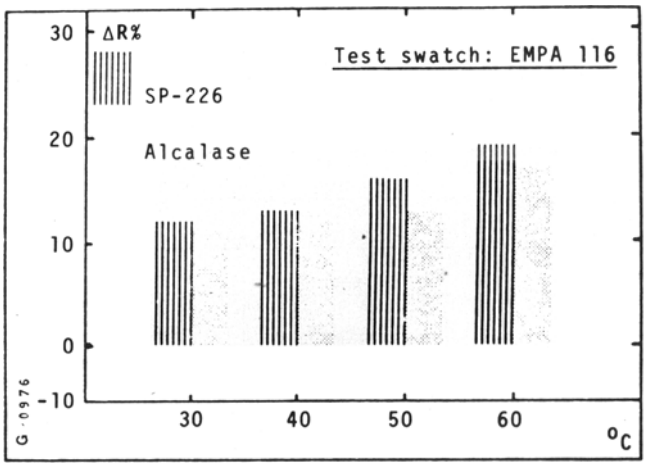

FIG. 15. Effect of temperature on detergent efficiency. U.S. wash (15 min/30, 40, 50 and 60 C) using Terg-O-Tometer. Test swatch: EMPA 116. 1.5 g U.S. heavy-duty detergent/ℓ. Enzyme concentration: 0.013 g/ℓ. Water hardness: 178 ppm/10°dH.

U.S. washing. The use of warm tap water for a relatively short time is characteristic for this washing method. The result of a Terg-O-Tometer experiment with washing at 50 C for 15 min is shown in Figure 10. It was found that SP 226 still had the best effect, but the difference between the two enzymes was not as pronounced as at 20 C. The test swatches egg/polyester-cotton and blood/acrylic responded well to SP 226. A repetition of this experiment in a full-size, top-loading machine was done (Fig. 11). The results confirm the findings in the Terg-O-Tometer test and further show that the problem with negative effect – soil redeposition – can be solved by using a more favorable detergent solution/test swatch ratio and better mechanical agitation.

European washing. In Europe, the washing normally starts with cold tap water, which, in the course of 45 min is heated to 40, 60 or 90 C. Most detergent products contain sodium perborate.

Figure 12 shows the results of a Launder-O-Meter experiment with a heavy-duty perborate-containing detergent in which the temperature was raised from 15 to 90 C in the course of 40 min. Toward denatured egg/cotton and spinach/cotton, Alcalase was slightly better than SP 226, which, however, had a much better effect on native egg soiling and blood.

The same pattern was observed in an experiment in which the temperature was raised to only 60 C (Fig. 13).

As already mentioned, these two experiments were done with a perborate-containing detergent and, therefore, it was natural to perform an experiment without perborate in order to evaluate the impact of the bleaching agent on the detergent efficiency. A European procedure was used and Figure 14 shows that the difference in detergent efficiency between the two enzymes has increased significantly. An extraordinarily good effect of SP 226 on the swatches with native egg soiling was observed.

Influence of temperature on detergent efficiency. The washing experiments just discussed indicate that the benefit of using SP 226 instead of Alcalase is more pronounced at moderate temperatures. In order to quantify the influence of temperature, a series of experiments were performed at 30, 40, 50 and 60 C. Apart from temperature, the washing conditions were identical to those used in the Terg-O-Tometer experiments discussed earlier. The swatches used

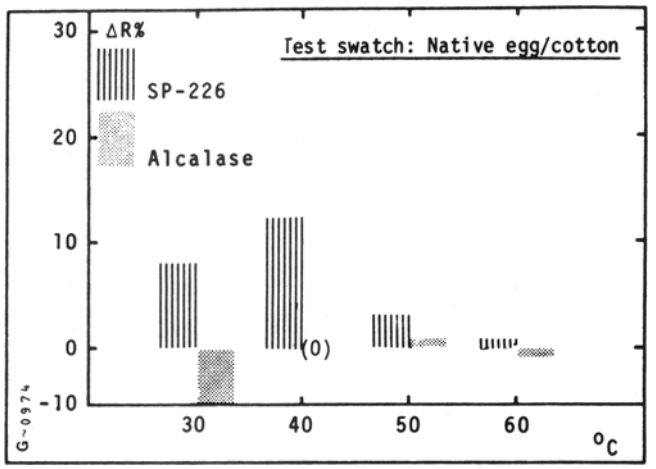

FIG. 16. Effect of temperature on detergent efficiency. U.S. wash (15 min/30, 40, 50 and 60 C) using Terg-O-Tometer. Test swatch: native egg/cotton. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.013 g/ℓ.
Water hardness: 178 ppm/10°dH.

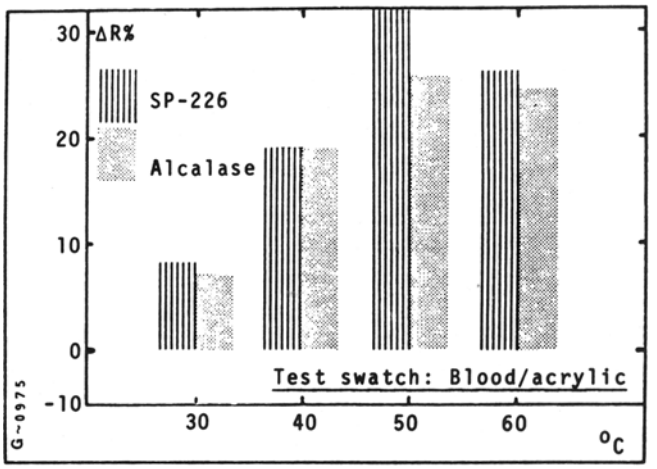

FIG. 17. Effect of temperature on detergent efficiency. U.S. wash (15 min/30, 40, 50 and 60 C) Test swatch: blood/acrylic. 1.5 g U.S. heavy-duty detergent/ℓ.
Enzyme concentration: 0.013 g/ℓ.
Water hardness: 178 ppm/10°dH.

were EMPA 116, native egg/cotton and blood/acrylic. With EMPA 116 (Fig. 15), the difference between the two enzymes is negligible, and for both, there might be a tendency to better performance at higher temperatures.

The outstanding effect of SP 226 on native egg soilings at a washing temperature below 40–45 C was again demonstrated (Fig. 16). Under these experimental conditions, Alcalase has a negligible effect and at temperatures below 40 C, even a negative one.

With blood/acrylic swatches (Fig. 17), a better effect of SP 226 was again observed and in this case, the optimal effect was found at 50 C.

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