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[54] **CHEMICALLY MODIFIED PROTEOLYTIC ENZYMES**

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[52] **U.S. Cl.** **195/63,** 195/62, 195/68, 252/89

[51] **Int. Cl.** **C07g 7/02**

[58] **Field of Search** 195/63, 68, 62, 66; 960/112; 252/89

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[57] **ABSTRACT**

Stabilized alkaline proteolytic enzymes having increased positive charge are prepared by contacting the native enzymes with compounds which attach additional basic and onium groups to the enzymes.

2 Claims, No Drawings

CHEMICALLY MODIFIED PROTEOLYTIC ENZYMES

BACKGROUND OF THE INVENTION

This invention is concerned with enzymes for detergent use. More particularly, this invention relates to chemical modification of alkaline proteolytic enzymes for use in detergent formulations.

Laundry products containing enzymes are old, being described, in for example, U.S. Pat. Specification No. 1,882,270. Enzymes aid in laundering by attacking soil and stains found on soiled fabrics whereby they are decomposed or altered in such an attack so as to render them more readily removable during laundering. Enzymes can be used in a soaking or pre-wash product designed to prepare soiled fabric for more effective detergency when the fabrics are subjected to conventional laundering, or as a component of a detergent formulation containing conventional cleaning ingredients.

The enzymes used in laundry products are solid catalytically active protein materials which degrade or alter proteinaceous soil or stains encountered in laundering situations. The enzymes are active in a pH range of from about 7 to about 11, and at a temperature of from about 50°F. to about 185°F.

The proteases catalyze the hydrolysis of the peptide linkage of proteins, polypeptides and related compounds to free amino and carboxyl groups and thus break down the protein structure in soil. Preferred proteases are those which are active in the neutral to alkaline pH range and are produced by microorganisms such as bacteria, fungi or molds.

SUMMARY OF THE INVENTION

In general, this invention is concerned with stabilized alkaline proteolytic enzymes for detergent use in which positive charge has been increased above that of the native alkaline proteases in the pH range of 7–11 by chemically incorporating additional basic and onium groups. These chemically modified enzymes are superior to native enzymes in soil removal at higher temperatures and pH's often encountered under typical household laundry situations where these factors are not closely controlled.

DETAILED DESCRIPTION OF THE INVENTION

This invention is concerned with the chemical modification of alkaline "proteolytic" enzymes used in laundry compositions. In particular, this invention relates to chemical modifications of these enzymes so as to provide improved cleaning of substrates soiled with proteinaceous stains at higher temperatures and pH's than presently used, e.g., 60°C and pH 10 or 50°C. and pH 11 with comparable performance to that of the native enzymes under commonly used conditions, 50°C. and pH 10. Higher pH improvement is desired for new detergent formulations which may benefit from more basic conditions such as formulation with decreased amounts of phosphate builder. Some improvement in package stability also results.

Alkaline proteases produced by microorganisms such as Bacillus subtilis, B. alcalophilus or Streptomyces rectus var. proteolyticus are treated with reagents which result in increased positive charge on the enzymes. These reagents are selected alkylating agents and agents which introduce the



group (R = alkyl, aryl, amino). Chemicals accomplishing these changes include methyl iodide, methyl bromide, ethylenimine, O-methylisourea and alkyl imido esters. Methyl iodide or methyl bromide are believed to react with amino and methylthio groups in the lysine and methionine residues of the enzymes to introduce alkylated and onium functions. Ethylenimine is believed to behave similarly, but additional amino groups are introduced from the ethylenimine. O-Methylisourea and alkyl imido esters are believed to convert lysine amino groups to amidine groups of greater basicity. In each of these types, the modified enzyme at a particular pH (7–11) has a greater positive charge, detectable by electrophoresis, by vitue of increased degree of protonation or onium formation in comparison with the native enzyme.

There are a number of enzyme products that lend themselves to the chemical modifications described in the process of this invention. Specific examples include the following: Maxatase (Pfizer Inc.), a subtilopeptidase A type enzyme from Bacillus subtilis strain R_4; Sigma VII (Sigma Chemical Co., St. Louis, Mo.), a subtilopeptidase B type enzyme from Bacillus subtilis; alkaline protease from Streptomyces rectus var. proteolyticus;and alkaline protease from Bascillus alcalophilus NCIB 8,772

The chemical reagents and reaction conditions described herein are well known to those skilled in the art of protein and enzyme chemistry.

Terg-o-tometer tests are used to determine the relative efficiency of enzyme systems in detergent formulations in removing protein, carbohydrate or fatty soils from synthetically soiled fabrics. The mechanical action in the Terg-o-tometer simulates that of a domestic type washer.

1. Equipment:

Terg-o-tometer- U.S. Testing Co., Inc-Hoboken, N.J. Photovolt Reflectometer- Photovolt Corp., New York, N.Y. Wringer, Motorized - Atlas Electric Devices Co., Chicago, Ill. Drum Drier - Premier Roto-Drier, Photo Mat'l Co., Chicago, Ill. Standard Soil Cloth- EMPA 116 (soiled with blood, milk, Japanese ink), Testfabrics, Inc. New York, N.Y.-swatches approximately 3 ½ inches × 4 inches, edges sewn with locking stitch to prevent ravelling.

2. a. Test Conditions

Detergent Concentration - 2 grams per liter

Test Temperature - 50°C, and 60°C

Enzyme Concentration - 500 DU to 2,000 DU per gram of detergent

Wash and Rinse Water- 1 liter synthetic tap water (STW) 50 ppm (50% Ca++, 40% Mg++)

Wash cycle- 1 ten minute wash

Rinse cycles- two 5 minute rinses

Agitator speed- 100 cpm.

b. Test Procedure

The terg-o-tometer bath is heated to constant temperature. One liter of STW is measured into each of the four beakers and equilibrated to test temperature with occasional agitation. Two grams of detergent are added to each beaker and the solutions agitated for 30 seconds. One of the wash solutions serves as a control containing the enzyme-free detergent while enzyme is added to the other three in the desired concentrations. The enzymes are usually added by aliquot from con-

centrated solution. Once the enzymes are added, the solutions are again agitated for 30 seconds. Four soiled swatches are individually placed in each beaker while agitating at 100 cpm. After a 10 minute wash cycle, the beakers are removed from the Terg-o-tometer, detergent solution is discarded and the washed cloths squeezed by hand. Excess wash solution is further removed by passing the swatches through the Atlas Wringer. The empty beakers are rinsed with lukewarm tap water and again charged with 1 liter of STW and equilibrated to temperature. Agitation is started, the cloths placed in their respective beakers as before, and rinsed for 5 minutes. Beakers are again removed, solution discarded, and cloths wrung out as before. This rinse cycle is repeated a second time. The washed and rinsed cloths are dried on the drum drier, then equilibrated in a constant temperature room at 72°F and 50% R.H. prior to making reflectance measurements.

3. Detergency Measurements

The reflectance of the swatches is measured before and after washing with the Photovolt Reflectometer employing a 610D search unit with green filter. Measurements are made relative to unsoiled (and unwashed) EMPA cloth in triple thickness, white standard. The dark side of the test cloth before washing, and both sides after washing are tested for reflectance. The average reflectance of the two sides after washing is used in the following equation to calculate percent Soil Removal (S.R.), where R= avg. reflectance of 4 swatches:

$$\% \text{ S.R.} = (\text{R washed cloths} - \text{R unwashed cloth}/\text{R white standard} - \text{R unwashed cloth}) \times 100$$

The Delft Unit (DU), referred to herein, is an arbitrary unit which is defined as follows: If one ml. of a 2% solution of an enzyme preparation gives a corrected absorbance of 0.400 under the test conditions, the enzyme preparation has a protease activity of 1,000 DU per gram.

The following examples are merely illustrative and are not intended to limit the invention, the scope of which is defined by the appended claims.

EXAMPLE I

To a solution of 0.5 g. of Maxatase, 1,241 DU/mg.,(Pfizer Inc, subtilopeptidase A from Bacillus subtilis strain R_4) in 50 ml. of deionized water is added 46 μl of ethylenimine. The resulting solution is stirred at room temperature for 24 hours, dialyzed against water for 4 hours at 10°C. and lyophilized to give 249 mg. of white powder. Determination of free amino groups by the ninhydrin procedure indicate that approximately 10 additional amino groups are present in the modified enzyme. In the soil removal test, the modified enzyme is significantly better than Maxatase at 60°C.

EXAMPLE II

The process of Example I is repeated with alkaline protease from Streptomyces rectus var. proteolyticus in place of Maxatase, with comparable results.

EXAMPLE III

The process of Example I is repeated with subtilopeptidase B (Sigma VII, Sigma Chemical Co., St. Louis, Mo.), with comparable results.

EXAMPLE IV

The process of Example I is repeated with alkaline protease from Bacillus alcalophilus NCIB 8,772 in place of Maxatase, with comparable results.

EXAMPLE V

To a solution of 1.0 g. of Maxatase (1,241 DU/mg.) in 45 ml. of deionized water is added 5 ml. of methyl iodide. The resulting mixture is titrated to pH 5 with 6 N HCl and stirred vigorously at room temperature for 24 hours. The resulting two layers are separated, the aqueous layer dialyzed against water at 5°C. for 6 hours and lyophilized to give 350 mg. of light brown powder. The modified enzyme is similar to Maxatase in the soil removal test at 50°C. and significantly better than Maxatase at 60°C.

EXAMPLE VI

The process of Example V is repeated with methyl bromide in place of methyl iodide, with comparable results.

EXAMPLE VII

The process of Example V is repeated with ethyl iodide in place of methyl iodide, with comparable results.

EXAMPLE VIII

A solution of 3.580 g. of ethyl acetamidate hydrochloride in 20 ml. of ice cold water is titrated to about pH 10 with 15% sodium hydroxide solution. To this cold solution is added 10.0 g. of Maxatase (890 DU/mg.) in 50 ml. of water. After stirring for 22.5 hours at 3°–5°C., the solution is dialyzed against water for 21.5 hours at 3°–5°C. and lyophilized to give 2.49 g. of white powder. The modified enzyme is similar to Maxatase in soil removal at 50°C. and significantly better than Maxatase at 60°C. The modified enzyme is also superior to Maxatase in the soil removal test at pH 11 and 50°C.

EXAMPLE IX

The process of Example VIII is repeated in turn with methyl acetamidate hydrochloride, propyl acetamidate hydrochloride, and butyl acetamide hydrochloride in place of ethyl acetamide hydrochloride, with comparable results.

EXAMPLE X

The process of Example VIII is repeated with alkaline protease from Streptomyces rectus var. proteolyticus in place of Maxatase, with comparable results.

EXAMPLE XI

The process of Example VIII is repeated with subtilopeptidase B from Bacillus subtilis in place of Maxatase, with comparable results.

EXAMPLE XII

The process of Example VIII is repeated with alkaline protease from Bacillus alcalophilus NCIB 8,772 in place of Maxatase, with comparable results.

EXAMPLE XIII

A solution of 2.0 g. of O-methylisourea hydrogen sulfate in 20 ml. of deionized water is titrated to pH 10 with 10% sodium hydroxide solution. To this is added

a solution of 4.0 g. of Maxatase (890 DU/mg) in 30 ml. of deionized water. After stirring for 20 hours at 3°–5°C., the cold solution is neutralized with 6 N HCl, dialyzed against water for 21.5 hours at 3°–5°C. and lyophilized to give 1.02 g. of white powder. The modified enzyme is superior in soil removal to Maxatase at 60°C. and about the same as Maxatase at 50°C.

EXAMPLE XIV

The process of Example XIII is repeated in turn with O-ethylisourea hydrogen sulfate, O-propylisourea hydrogen sulfate and O-butylisourea hydrogen sulfate in place of O-methylisourea hydrogen sulfate, with comparable results.

EXAMPLE XV

The process of Example XIII is repeated with subtilopeptidase B from Bacillus subtilis in place of Maxatase, with comparable results.

EXAMPLE XVI

The process of Example XIII is repeated with alkaline protease from Streptomyces rectus var. proteolyticus in place of Maxatase, with comparable results.

EXAMPLE XVII

The process of Example XIII is repeated with alkaline protease from Bacillus alcalophilus NCIB 8,772 in place of Maxatase, with comparable results.

EXAMPLE XVIII

A solution of 5.38 g. of ethyl imidobenzoate hydrochloride (prepared according to the procedure of S.M.McElvain, Jour. Am. Chem. Soc. 68, 1917–1921 (1946) in 20 ml. of cold deionized water is adjusted to pH 10 with 10% sodium hydroxide solution in an ice bath. To this solution is added 10.0 g. of Maxatase (890 DU/mg) and enough cold water to bring the solution to a volume of about 50 ml. This solution is stirred in a cold room at 5°–10°C. for 23 hours. It is diluted to 200 ml. with water, dialyzed in regenerated cellulose tubing against 6 liters of water in the cold room and lyophilized to yield 3.38 g. of the modified enzyme product. In the soil removal test the modified enzyme is superior to Maxatase at 50°C. and pH 11, and equivalent at 50°C. or 60°C. and pH 10.

EXAMPLE XIX

The process of Example XVIII is repeated with subtilopeptidase B from Bacillus subtilis in place of Maxatase, with comparable results.

EXAMPLE XX

The process of Example XVIII is repeated with alkaline protease from Bacillus alcalophilus NCIB in place of Maxatase, with comparable results.

EXAMPLE XXI

The process of Example XVIII is repeated with alkaline protease from Streptomyces rectus var. proteolyticus in place of Maxatase, with comparable results.

What is claimed is:

1. A stabilized enzyme with increased positive charge for detergent use prepared by contacting under reaction conditions the native enzyme subtilopeptidase B from Bacillus subtilis with
   ethylenimine
   methyl iodide
   ethyl iodide
   methyl bromide
   methyl acetamidate hydrochloride
   ethyl acetamidate hydrochloride
   propyl acetamidate hydrochloride
   butyl acetamidate hydrochloride
   o-methylisourea hydrogen sulfate
   o-ethylisourea hydrogen sulfate
   o-propylisourea hydrogen sulfate
   o-butylisourea hydrogen sulfate
   or
   ethyl imidobenzoate hydrochloride.

2. A stabilized enzyme with increased positive charge for detergent use prepared by contacting under reaction conditions the native enzyme alkaline protease from Streptomyces rectus var. proteolyticus with
   ethylenimine
   methyl iodide
   ethyl iodide
   methyl bromide
   methyl acetamidate hydrochloride
   ethyl acetamidate hydrochloride
   propyl acetamidate hydrochloride
   butyl acetamidate hydrochloride
   o-methylisourea hydrogen sulfate
   o-ethylisourea hydrogen sulfate
   o-propylisourea hydrogen sulfate
   o-butylisourea hydrogen sulfate
   or
   ethyl imidobenzoate hydrochloride.

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