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(54) **Protein stain-removing compositions.**

(57) Proteinaceous stains are removed more effectively from fabrics by treatment with protease in the presence of a reagent which cleaves disulfide bonds. A detergent is preferably also used.

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Description

PROTEIN STAIN-REMOVING COMPOSITIONS

Technical Field

The invention relates to the removal of stains using detergent compositions. More specifically, compositions which are capable of removing protein-containing stains, such as blood and food, are disclosed.

Background Art

It is well established that the addition of proteases to laundry detergents has a beneficial effect. Desirably, the proteases used will function at the relatively high pH values (7.5-10.5) that are conducive to the performance of commonly used detergents. Typical commercial detergents rely for their cleaning power on highly basic substances such as trisodium phosphate, detergent substances such as the long-chain alkyl or aryl sulfonates, and the protease additives such as subtilisin or other proteolytic enzymes.

It is a familiar phenomenon that while detergent compositions have become increasingly effective over the years, they fail to remove efficiently certain protein-containing stains, notably blood stains. Indeed, blood stains are notoriously resistant to removal, especially after remaining in the fabric for some time.

The compositions of the present invention provide a more effective release of protein-containing stains by causing disulfide bond cleavage in addition to proteolysis in the context of a detergent preparation. The combination of proteolytic enzymes with disulfide cleavage reagents has been used previously in an effort to destroy proteins in research preparations, such as in the preparation of nucleic acids, or to fragment the target proteins, as in the performance of Cleveland gels for mapping peptides. Use of a combination of a disulfide cleaving reducing agent, such as calcium thioglycolate with a protease to dissolve hair in clogged drains was disclosed in US 4,540,506. Up to 10% detergent could be added to the compositions used for this purpose. U.S. patent 4,294,087 describes a method to dissociate and recover hair from animal hides using successive cleavage of disulfide bridges followed by protease treatment. British patent application GB 2,139,260A, published 7 November 1984, discloses the simultaneous use of peroxidant bleach and a protease in detergent compositions, wherein the presence of manganese ion permitted this composition to remain effective at lower temperature range than those previously used. According to this disclosure, without the inclusion of metal ion catalysts in the composition, decomposition of the peroxide and action of the bleach requires temperatures above 70°C; even in the presence of these ions (other than manganese), temperatures above 60°C are required. The manganese ion-containing compositions disclosed in the British patent may contain up to 50% detergent.

To Applicants' knowledge, the combination of a protease and a disulfide cleavage reagent has never been used as an additive to a detergent composition for removal of proteinaceous materials from fabrics, or in detergent compositions operable for this purpose at low temperature.

Disclosure of the Invention

The invention provides an effective method and detergent composition for the removal of blood, food, and other protein-containing stains from fabrics, regardless of whether washing is prompt or delayed over a period of days or weeks. The composition utilizes ordinary detergent preparations, but with the addition of both a protease and effective amount of a substance capable of cleaving disulfide bonds. The resulting preparations are operable at temperatures of less than 70°C and even at temperatures below 55°C.

In one aspect, the invention is directed to a detergent composition which comprises an effective amount of a protease and of a substance capable of cleaving disulfide bonds (a disulfide cleavage reagent or DCR) in admixture with a suitable detergent formulation. In another aspect, the invention relates to a process for removing proteinaceous stains from fabrics using protease and disulfide cleavage reagent or the compositions of the invention. The process is effective at a temperature below 70°C, and does not require metal ions. In still another aspect, the invention relates to methods for preparing the detergent compositions.

Brief Description of the Drawings

Figure 1 shows a comparison of a stained cloth swatch washed using the method of the invention with similar swatches washed with detergent alone, with detergent plus protease alone, or with detergent plus cleavage reagent alone.

Modes of Carrying Out the Invention

In the aspect of the invention directed to compositions useful for removing protein-containing stains, the essential components of the composition are a protease, a substance or mixture of substances capable of cleaving disulfide bonds, and a detergent formulation carrier.

The protease is one of, or a mixture of general specificity proteolytic enzymes which are available commercially or otherwise are known in the art. While in theory any protease can be used, more favorable embodiments are those which have pH optima suitable for use in the presence of the mildly basic environments associated with detergent compositions. Thus, while pepsin and papain, for example, are marginally workable, enzymes with higher pH optima such as trypsin, chymotrypsin, subtilisin, and the like, are

preferred. The preferred pH range is 7-12, more preferably 10-12. A number of commercially available enzyme preparations useful in detergents are also appropriate. Such enzymes include those trademarked Alcalase, Maxatase, Maxacal, and Esperase. Particularly preferred is the subtilisin prepared from Bacillus amyloliquefaciens, disclosed in European patent application publication no. 130,756, published 9 January 1985. The compositions of the invention may include any protease or mixtures thereof, and the most effective choice will depend on the conditions of use, including pH, temperature, length of wash time, and the presence or absence of particular inhibitors with respect to the subject enzyme.

Substances capable of cleaving disulfide bonds are varied, but fall generally into three categories: oxidizing agents, reducing agents, and miscellaneous addition substrates such as those exemplified by fumaric acid and sodium sulfite. Suitable oxidizing agents include hydrogen peroxide, performic acid, sodium perborate, and oxidizing bleaches. Effective reducing agents include dithiothreitol (DTT), β-mercaptoethanol (BME), sodium borohydride, and the like.

Alternate reagents which are not easily classified include mercuric chloride, nitroprusside, tributylphosphine, and phosphothiolate. A particularly useful cleavage reagent is sodium sulfite, while results in sulfitolysis of the disulfide according to the reaction: $R\text{-}S\text{-}S\text{-}R + SO_3^{-2} \rightarrow R\text{-}S\text{-}SO_3^{-2} + -SR$. The equilibrium of this reaction may be shifted by removal of the thiol anion using heavy metal ions or oxidizing agents. The oxidizing power may be provided by aeration if the wash solution is agitated, as is typically the case in conventional washing machines, or an oxidizing agent, such as $CuSO_4$ or sodium perborate, may be added.

The foregoing list of substances capable of cleaving disulfides is not meant to be comprehensive, and conversely does include substances which are effective but not necessarily appropriate for a commercial product. In order to be successful commercially, the added substance must be relatively inexpensive and must not have undesirable properties. Thus, for example, while the use of mercuric chloride would be workable in carrying out the process of the invention, it would not be suitable for ordinary detergent products intended for household use. β-mercaptoethanol and DTT are feasible commercially, except that they have mildly offensive odors and would need to be disguised by perfume in commercial preparations. Particularly preferred substances, therefore, for commercial detergents, are sodium sulfite (preferably in combination with an oxidizing agent) or hydrogen peroxide, which are inexpensive and are relatively safe. Reviews of materials which are useful in the cleavage of disulfide bonds are found, for example, in Chemical Modification of Proteins, Means, G.E., et al, eds (1971), Holden-Day, Inc, San Francisco, CA, Ch 8; and Chemical Reagents for Protein Modification, Lundbald, R.L., et al, eds (1984), CRC Press Inc, Boca Raton, FL, Ch 7.

The remainder of the composition is that of a conventional detergent formulation, typically containing a base such as trisodium phosphate, detergents such as linear alkyl benzene sulfonates, alkyl ethoxylated sulfate, sulfated linear alcohol or ethoxylated linear alcohol, or soap, and other components such as builders and whiteners. As is known, detergent compositions may be in solid, granular, or liquid form. Of course, mixtures of bases and detergents can be used, as well. Typical detergent compositions are disclosed in, for example, U.S. patents 3,623,957; 4,404,128; 4,381,247; 4,404,115; 4,318,818; 4,261,868; 4,242,219; 4,142,999; 4,111,,855; 4,011,169; 4,090,973; 3,985,686; 3,790,482; 3,749,671; 3,560,392; 3,558,498; and 3,557,002.

Typically the protease forms 0.01- 3% wt/wt of the detergent compositions of the invention, and the disulfide-cleaving substance, 10-40% wt/wt thereof. The amounts present depend, of course, on the nature of the protease and of the disulfide cleavage reagent, the dilution of the detergent in the wash solution, and the conditions of wash. However, the ranges given are generally typical.

In the method of the invention, fabrics containing proteinaceous stains are treated with the combination (simultaneous or sequential) of a protease and disulfide cleaving reagent at suitable pH, temperature, and time of wash. These conditions are, of course, variable according to convenience, and the selection of the protease and the substance to cleave disulfides to some extent depends on this selection. However, convenient conditions frequently encountered are pH values between 7 and 12, particularly at the high pH end of this range, at pH 10-12. Temperatures of 20°C-55°C, particularly around 40-55°C, and times of 5-20 minutes, usually around 10-15 minutes are typical and preferred. The preferred times and temperatures are those generally utilized in household washing machines, neighborhood laundromats, and professional laundry services, since in order to be commercially practical, the process needs to be conducted under conditions ordinarily available to the user. It is to be noted that despite the temperature range here employed (less than 70°C, and even 55°C and below), no heavy metal ion catalyst, such as Mn(II), is required or desirable.

In one embodiment of the process of the invention, conventional washing procedures using commercial detergents are used and the protease and disulfide-cleaving substance are provided, either separately or together, as an additive, much in the manner of the methods in which bleach is used. Thus, these may be added along with the detergent at the beginning of the wash cycle or at some intermediate point, for example, after approximately half of the wash cycle has been completed. If handled in this way, assuming an approximately 1:500 dilution of a solid detergent composition (approximately 2 mg/ml of the solid), arbitrary amounts of the protease and DCR may be added without the upper limit imposed by the dilution. (If the protease and DCR had been added to the detergent composition originally, and if, for example, the DCR constituted 50% of the composition, only 1 mg/ml would result in the final wash solution. However, if these materials are added separately, amounts most effective for the particular DCR and protease may be added.)

Limitation on amount is generally not a consideration with respect to the protease, since only very small quantities are required. Typically, the protease is added to a final concentration of approximately 1-50 μg/ml of wash solution. In the case of the DCR, however, larger amounts than would be permitted by the dilution of the

detergent may be desirable. For example, cleavage of disulfide bonds using sodium borohydride may conveniently be carried out with concentrations as high as 0.2 M reagent in the present of similar quantities of buffer (Lundbald, R.L., et al, Chemical Reagents for Protein Modification, supra).

Although such high amounts are conventional, they are not necessarily required, and lower concentrations are workable. Sulfitolysis is ordinarily carried out in sodium sulfite concentrations of the order of 0.1 M, although concentrations as low as 0.01 M and lower can also be used. DTT is effective when supplied at concentrations of the order of 0.02-0.1 M. In short, the DCR concentration can be varied over a wide range for any of these reagents and effectiveness maintained. The optimum concentration for a particular application will, of course, depend on the nature of the stain and the nature of the reagent, as well as the conditions of the wash procedure, including time, temperature, and pH.

In an alternative and more convenient approach, the protease and disulfide-cleaving substance are added to the original detergent composition, and the process is conducted as a standard wash procedure using these modified detergents. Under these circumstances, the detergent composition will correspond to that described above, but the amount of the composition can also be varied over the range of approximately 0.5 mg/ml-10 mg/ml or greater of the wash solution, depending, again, on the conditions of the wash solution and procedure, and on the solubilities of the detergent components. In any case, the inclusion of the DCR and protease in the detergent limits the concentrations of these components in accordance with the dilution of the detergent. Thus, even if a 1:100 dilution is used (10 mg/ml), and the DCR, for example, is limited to 50% of the detergent composition, a maximum concentration of 5 mg/ml DCR in the resulting wash solution is an upper limit. For a hypothetical molecular weight of 100 mg/mM, this results in a concentration of .05 M maximum for the DCR. Typically, of course, the concentration of DCR in the detergent will be less than 50%, mandating even lower concentration of the DCR.

The detergent compositions of the invention contain mostly detergent, relatively smaller amounts of DCR, and quite small amounts of protease, which is especially desirable in view of the cost of enzymic components. Thus, in general, the preparation will contain 60-90% "detergent" (surface active substances but including the additives such as builders and whiteners), 0.01-3% protease, and approximately 10-40% disulfide cleavage reagent.

Of course, it is also possible to add only one of these two additives to the original detergent and to supply the other separately to the wash liquid. In particular, the disulfide cleavage reagent may be added to a prewash, followed by a detergent containing the protease, or addition of the detergent containing disulfide cleavage reagent may be followed or preceded by treatment with protease. The following protocols are exemplary:

Example	Cycle 1	Cycle 2
1	Detergent (0.1% protease) 10 min, 37°C	Detergent (10% DCR) 10 min, 37°C
2	30 mM DCR 15 min, 30°C	Detergent (0.1% protease) 15 min, 30°C
3	Detergent (0.2% protease, 20% DCR)	---
4	Detergent (0.1% protease, 25% DCR) 10 min, 37°C	Detergent (0.2% protease) 10 min, 37°C
5	10µg/ml protease 10 min, 37°C	Detergent (10% DCR) 12 min, 30°C
6	Detergent (10% DCR) 12 min, 30°C	10 µg/ml protease 10 min, 37°C
7	100 mM DCR 15 min, 37°C	Detergent (0.2% protease) 10 min, 30°C
8	100 mM DCR, 5 µg protease 15 min, 37°C	Detergent 10 min, 30°C

The following examples show the results of laboratory testing which establishes the efficacy of the

procedure. These examples are also intended to illustrate but not to limit the invention.

Example 9

Increase of absorbance is a measure of protein extracted from test scratches by the test reagent containing disulfide-cleaving β-mercaptoethanol (BME) or dithiothreitol (DIT) using methods known in the art. The results are shown in Table 1 below.

Table 1

Enzyme (µg)	DCR	A280 Before Wash	A280 After Wash	A280 Change	Prot Rmng on Cloth (µg)
–	–	0.10	0.32	0.22	120
15	–	0.12	0.52	0.40	24
0	12mM BME	0.12	0.41	0.29	82
15	12mM BME	0.13	0.69	0.56	18
15	8mM DTT	0.13	0.71	0.58	8

(Results for DTT alone are not included because oxidized DTT absorbance at 280 nm interferes with interpretation of the data.)

The supernatants from the precipitate in the above test were disgarded and the protein content of the pellets quantitated using the method of Lowry (Lowry, O.H., et al, J Biol Chem (1951) 193:263-275) using BSA as the standard. The results of this assay are also shown in Table 1. The data indicate that the combination of protease and disulfide-cleaving reagent results a nearly complete removal of protein from the cloth, while either alone is relatively ineffective.

Example 10

Likewise, stained samples were incubated for 5 min at 95° C in solubilization buffer to extract the proteins for assay according to the Lowry procedure (supra).

The results of addition of protease (subtilisin) alone, disulfide cleavage reagent alone, and of the addition of both are shown in Table 2.

Table 2

Sample	Additions/Deletions	µg Protein Remaining
1	Delete Tide	127
2	None	78
3	10 µg subtilisin	5
4	10 mg Na_2SO_3	65
5	10 mg Na_2SO_3 + 10 µg subtilisin	3*
6	2 mM $CuSO_4$	147†
7	2 mM $CuSO_4$ + 10 µg subtilisin	35†
8	10 mg Na_2SO_3 + 2 mM $CuSO_4$	115
9	10 mg Na_2SO_3 + 2 mM $CuSO_4$ + 10 µg subtilisin	7
10	5 mg Na dithionite	43
11	5 mg Na dithionite + 10 µg subtilisin	2
12	10 mg Na_2SO_3 + 5 mg Na-dithionite	51
13	10 mg Na_2SO_3 + 5 mg Na-dithionite + 10 µg subtilisin	2
14	20 mM DTT	65
15	20 mM DTT + 10 µg subtilisin	2
16	50 µg sodium perborate	44
17	50 µg sodium perborate + 10 µg subtilisin	2

* Protein content lower than that achieved with subtilisin only was not consistently obtained.

† Addition of $CuSO_4$ either as an oxidizing agent directly or in conjunction with a sulfitolysis reagent interferes with the Lowry assay. See Example 12 for effect as asseyed by visual evidence.

As shown in Table 2, the combinations of detergent, protease, and disulfide cleavage reagent results in more effective removal of protein than detergent with either protease or DCR alone.

Example 11

The conditions of treatment (water wash, incubation, rinse, protein determination) were as in Example 9, but these samples had a high initial protein content. The results are shown in Table 3.

Table 3

Samples	Additions/Deletions	µg Proteins Remaining
1	Delete Tide	270
2	None	170
3	1 mg Na_2SO_3 + 2 mM $CuSO_4$	260
4	1 mg Na_2SO_3 + 2 mM $CuSO_4$ + 10 µg subtilisin	5
5	25 mM DDT	170
6	25 mM DDT + 10 µg subtilisin	4
7	50 µg perborate	225
8	50 µg perborate + 10 µg subtilisin	2
9	1 mg Na_2SO_3 + 50 µg Na perborate	170
10	1 mg Na_2SO_3 + 50 µg Na perborate + 10 µg subtilisin	2
11	50 µg Na perborate pretreatment without Tide, then 10 µg subtilisin + Tide	3

Example 12

The protocol was as in Example 9, except that after rinse the swatches were dried on paper towels and photographed. The results are shown in Figure 1, and listed below.

Sample	Additions/Deletions
1	Delete Tide
2	None
3	10 µg subtilisin
4	20 mM DTT
5	20 mM DTT + 10 µg subtilisin
6	10 mg Na_2SO_3 + 2 mM $CuSO_4$
7	10 mg Na_2SO_3 + 2 mM $CuSO_4$ + 10 µg subtilisin
8	50 µg Na-perborate
9	50 µg Na-perborate + 10 µg subtilisin
10	5 mg Na-dithionite
11	5 mg Na-dithionite + 10 µg subtilisin

Reference to Figure 1 shows that Samples 5, 7, 9, and 11, which contain Tide, protease, and DCR, are noticeably better extracted than the controls containing subtilisin alone (Sample 3) or DCR alone (Samples 4, 6, 8, 10).

Claims

1. A method for removing proteinaceous stains from a fabric which comprises treating said fabric with an effective amount of protease and with an effective amount of a reagent capable of cleaving disulfide bonds (a disulfide cleavage reagent, DCR).
2. A method according to claim 1 wherein treating with protease and treating with DCR is conducted sequentially in steps.
3. A method according to claim 1 or 2 which further includes treating the fabric with detergent.
4. A method according to any one of the preceding claims wherein the protease and DCR are contained in a wash solution.
5. A method according to claim 4 wherein the wash solution further contains detergent.
6. A method according to any one of the preceding claims carried out at a temperature of from 20°C to less than 70°C.
7. A method according to claim 6 carried out at 20° to 55°C.
8. A method according to any one of the preceding claims carried out at pH 7 to 12.
9. A method according to any one of the preceding claims wherein the protease is subtilisin.
10. A method according to claim 9 wherein the subtilisin is derived from B. amyloliquefaciens.
11. A method according to any one of the preceding claims wherein the DCR comprises a sulfitolysis reagent.
12. A method according to claim 11 wherein the sulfitolysis reagent is sodium sulfite.
13. A method according to any one of the preceding claims wherein the DCR further contains a reagent reactive with thiol anions of the formula RS- wherein R is an organic radical.
14. A method according to claim 13 wherein said reagent reactive with thiol anions is an oxidizing agent.
15. A method according to claim 14 wherein the oxidizing agent is selected from sodium perborate, sodium dithionite and copper sulfate.
16. A method according to any one of claims 1 to 10 wherein the DCR is a reducing agent.
17. A method according to claim 16 wherein the reducing agent is beta-mercaptoethanol or dithiothreitol.
18. A method according to any one of claims 1 to 10 wherein the DCR is an oxidizing agent.
19. A method according to claim 18 wherein the oxidizing agent is selected from sodium perborate and sodium dithionite.
20. A detergent composition which comprises an effective amount of a protease and of a substance capable of cleaving disulfide bonds (disulfide cleavage reagent, DCR) in admixture with a detergent formulation.
21. A composition according to claim 20 wherein the protease is subtilisin.
22. A composition according to claim 21 wherein the subtilisin is derived from B. amyloliquefaciens.
23. A composition according to any one of claims 20 to 22 wherein the DCR comprises a sulfitolysis reagent.
24. A composition according to claim 23 wherein the sulfitolysis reagent is sodium sulfite.
25. A composition according to any one of claims 20 to 24 wherein the DCR further contains a reagent reactive with thiol anions of the formula RS- wherein R is an organic radical.

26. A composition according to claim 25 wherein said reagent reactive with thiol anions is an oxidizing agent.

27. A composition according to claim 26 wherein said oxidizing agent is selected from sodium perborate, sodium dithionite and copper sulfate.

28. A composition according to any one of claims 20 to 22 wherein the DCR is a reducing agent.

29. A composition according to claim 28 wherein the reducing agent is beta-mercaptoethanol or dithiothreitol.

30. A composition according to any one of claims 20 to 22 wherein the DCR is an oxidizing agent.

31. A composition according to claim 30 wherein the oxidizing agent is selected from sodium perborate and sodium dithionite.

32. A composition according to any one of claims 20 to 31 wherein the detergent is 60-90% by weight of said composition, the protease is 0.01-3% by weight of said composition and the disulfide cleavage reagent is 10-40% by weight of said composition.

FIG. 1

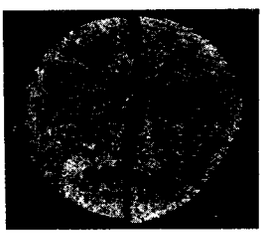

FIG. 2

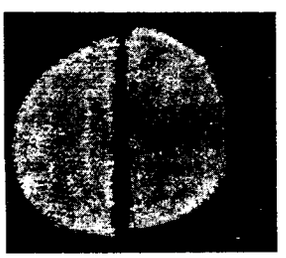

FIG. 3

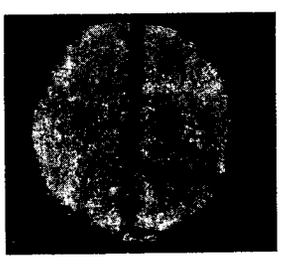

FIG. 4

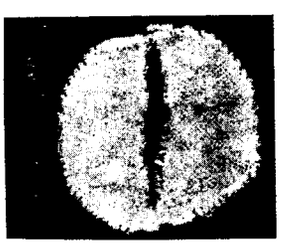

FIG. 5

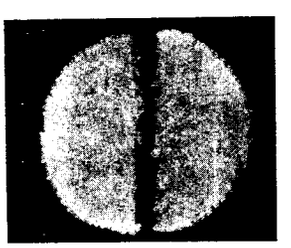

FIG. 6

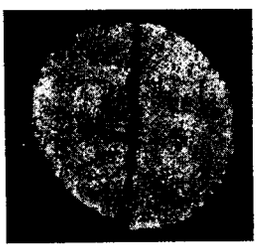

FIG. 7

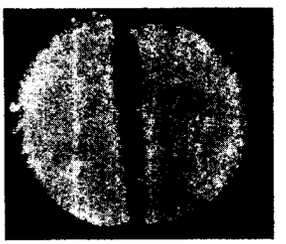

FIG. 8

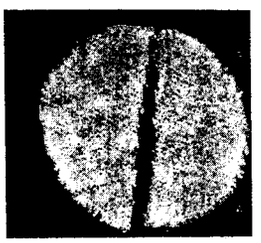

FIG. 9

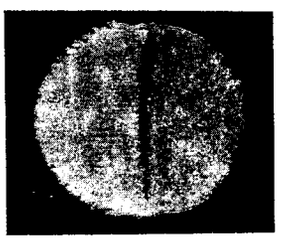

FIG. 10

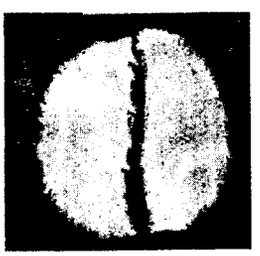

FIG. 11