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Methods for rapidly digesting biopolymers with ultrastable enzymes for mass spectrometry-based analyses

Abstract

The present disclosure relates to a composition containing an ultrastable enzyme, methods of using the same for preparing a biological sample for analysis by mass spectrometry, and kits comprising the same. The composition includes an ultrastable enzyme isolated from a hyperthermophilic and/or acidophilic organism and optionally, an acid and an additive. The composition can be used at temperatures ranging from about 50° C. to 110° C., preferably at temperatures ranging from about 70° C. to 100° C. In addition, the composition can be used at pH values ranging from 0.5 to 7, preferably at pH values ranging from 2 to 5.

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Background/Summary

CROSS-REFERENCE TO RELATED APPLICATIONS (1) This application is a national stage of international application no. PCT/US2018/057397, filed Oct. 24, 2018, which claims the benefit of U.S. provisional application No. 62/576,374 filed Oct. 24, 2017, the contents of each of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

(1) The present disclosure generally relates to compositions comprising a thermally and/or acid stable enzyme and optionally, an acid, detergent, alkylating agent, and/or other chemical, and methods of using the same, for preparation of samples for proteomic, glycomic, glycoproteomic, or other chemical, biochemical, or immunochemical analyses.

REFERENCE TO SEQUENCE LISTING

(2) This application includes an electronically submitted sequence listing in .txt format. The .txt file contains a sequence listing entitled “15797.00017_ST25.txt” created on May 27, 2025, and is 159,472 bytes in size. The sequence listing contained in this .txt file is part of the specification and is hereby incorporated by reference herein in its entirety.

BACKGROUND

(3) Proteins are essential cellular machinery, performing and enabling tasks within biological systems. The variety of proteins is extensive, and the role they occupy in biology is deep and complex. Each step of cellular generation, from replication of genetic material to cell senescence and death, relies on the correct function of several distinct proteins. The precision of cellular machinery can be disrupted, however, resulting in disease. Because much of the machinery essential to cell health and survival remains unknown, studying proteins is of great interest and importance.

(4) Proteomics involves the large-scale study of proteins and their ability to regulate cellular functions, including analyzing their presence, modification status, and quantities in biological samples. The field of proteomics encompasses many techniques, such as immunoassays and two-dimensional differential gel electrophoresis (2-D DIGE). Another group of methodologies that are growing in popularity for protein discovery and analyses are mass spectrometry-based approaches. However, in circumstances where biological samples are mass-limited, obtaining sufficient quantities of proteins to generate high-quality mass spectrometric data can pose a challenge. The quality and interpretation of proteomic analyses depend largely on the amount and nature of the proteins to be analyzed. The modification status and inherent nature of the proteins under study pose limitations to these types of analyses. Thus, sample preparation approaches that are time-consuming, or worse, fail to digest the target proteins or incur massive sample losses, are intolerable. There is a thus a need for techniques to prepare limited quantities of biological sample for analysis by mass spectrometry that are rapid, overcome existing limitations, and preserve protein quantities in the sample without large sample loss.

(5) Current mass spectrometry-based analyses face technical limitations that are primarily due to limitations of the enzymes used for biomolecule digestion. Target biopolymer digestion times, digestion completeness, and enzyme compatibility with chemical reagents are all limiting factors in the state-of-the-art procedures and limit throughput and quality of biomolecule analyses. Accordingly, disclosed herein are ultrastable enzymes that address these issues and offer novel capabilities to modern proteomic, lipomic, glycomic, and glycoproteomic approaches.

SUMMARY

(6) Provided herein are methods of preparing a biological sample, wherein the method includes: (a) providing the biological sample containing at least one biopolymer; (b) contacting the sample with a composition containing an ultrastable enzyme to form a reaction mixture; and (c) incubating the

reaction mixture for at least one second, resulting in the digestion or modification of the at least one biopolymer present in the biological sample. In some embodiments, the biological sample can be prepared for mass spectrometry-based proteomic analysis, glycomic analysis, glycoproteomic analysis, lipomic analysis, amino acid analysis, enzymatic analysis, or immunochemical analysis.
(7) In some embodiments, the biological sample is one selected from the group consisting of: a tissue, a cell pellet, a cell lysate, a cell culture solution, a biological fluid, a plant tissue, a plant fluid, a food product, an environmental sample, a gel sample and the like.
(8) In some embodiments, the composition containing the ultrastable enzyme further includes one or more agents selected from the group of: a detergent, an acid, an oxidizer, a surfactant, an additive for biopolymer digestion, a reactive and/or chaotropic chemical component, and mixtures thereof.
(9) In some embodiments, the composition containing the ultrastable enzyme further includes an acid. In some embodiments, the acid is selected from the group consisting of: nitric acid, phosphoric acid, hydrofluoric acid, sulfuric acid, hydrochloric acid, acetic acid, paracetic acid, citric acid, glycolic acid, formic acid, and mixtures or combinations thereof.
(10) In some embodiments, the composition containing the ultrastable enzyme further includes a surfactant or detergent. In some embodiments, the surfactant or detergent is selected from the group consisting of: CHAPS, Big CHAP, CHAPSO, NP-40, sodium dodecyl sulfate (SDS), polysorbate 20 (Tween 20), polysorbate 80 (Tween 80), Triton® X-100, octyl glucoside, octyl thioglucoside, deoxycholate, and mixtures or combinations thereof.
(11) In some embodiments, the composition containing the ultrastable enzyme further includes an additive for biopolymer digestion or biopolymer modification. In some embodiments, the additive is selected from the group consisting of: iodoacetamide (IAA), dithiothreitol (DTT), RapiGest SF, PPS Silent® Surfactant, Invitrosol™, ProteaseMAX™, and mixtures or combinations thereof.
(12) In some embodiments, the ultrastable enzyme is isolated from an organism of the Archaea domain. In some embodiments, the ultrastable enzyme is isolated from an organism of the Sulfolobales order.
(13) In some embodiments, the ultrastable enzyme is is selected from the group consisting of: a protease, a lipase, a cellulase, a hemicellulase, a glycoside hydrolase, an endoprotease, a carboxyesterase, an amylase, an alpha-amylase, an endoglucanase, an endopullulanase, a PNGase, a trehalase, a pullulanase, a peptidase, a signal peptidase, a xylanase, a cellobiohydrolase (CBH), a β-glucosidase, a peroxidase, a phospholipase, an esterase, a cutinase, a pectinase, a pectate lyase, a mannanase, a keratinase, a reductase, an oxidase, a phenoloxidase, a lipoxygenase, a ligninase, a tannase, a pentosanase, a malanase, a Q-glucanase, an arabinosidase, a hyaluronidase, a chondroitinase, a lactase, a xyloglucanase, a xanthanase, an acyltransferase, a galactanase, a xanthan lyase, a xylanase, an arabinase, a glycohydrolase, a glycosyltransferase, a glycosidase, and combinations thereof.
(14) In any of the foregoing embodiments, the reaction mixture in step (c) can be incubated at a temperature of at least 50° C. In some embodiments, the reaction mixture in step (c) is incubated at at a temperature of from about 50° C. to about 150° C. In some embodiments, the reaction mixture in step (c) is incubated at a temperature of from about 60° C. to about 125° C. In some embodiments, the reaction mixture in step (c) is incubated at a temperature of from about 70° C. to about 100° C. In some embodiments, the reaction mixture in step (c) is incubated at a temperature of from about 75° C. to about 90° C. In some embodiments, the reaction mixture in step (c) is incubated at a temperature of from about 75° C. to about 85° C. In some embodiments, the reaction mixture in step (c) is incubated at a temperature of from about 75° C. to about 80° C.
(15) In any of the foregoing embodiments, the reaction mixture in step (c) can be incubated at a pH of from about 0.5 to about 7.0.
(16) In some embodiments, the reaction mixture in step (c) is incubated at a pH of from about 0.5 to about 4.5. In some embodiments, the reaction mixture in step (c) is incubated at a pH of from

about 0.5 to about 3.0. In some embodiments, the reaction mixture in step (c) is incubated at a pH of from about 0.5 to about 1.5.
(17) In some embodiments, the reaction mixture in step (c) is incubated at a pH of from about 4 to about 7. In some embodiments, the reaction mixture in step (c) is incubated at a pH of about 5.5. In some embodiments, the reaction mixture in step (c) is incubated at a pH of about 3.0.
(18) In some embodiments, the reaction mixture in step (c) is incubated for less than 8 hours, less than 4 hours, less than 2 hours, less than 1 hour, less than 45 minutes, less than 30 minutes, less than 15 minutes, less than 10 minutes, less than 5 minutes, less than 1 minute, less than 30 seconds, or less than 10 seconds.
(19) In some embodiments, the reaction mixture in step (c) is incubated for a duration of time ranging from about 5 minutes to about 300 minutes. In some embodiments, the reaction mixture in step (c) is incubated for a duration of time ranging from about 10 minutes to about 150 minutes. In some embodiments, the reaction mixture in step (c) is incubated for a duration of time ranging from about 20 minutes to about 90 minutes. In some embodiments, the reaction mixture in step (c) is incubated for a duration of time ranging from about 30 minutes to about 75 minutes. In some embodiments, the reaction mixture in step (c) is incubated for a duration of time ranging from about 40 minutes to about 60 minutes.
(20) In some embodiments, the reaction mixture in step (c) is incubated for a duration of time ranging from about 1 second to about 120 minutes, or from about 30 seconds to about 100 minutes, or from about 1 minute to about 90 minutes, or from about 10 minutes to about 75 minutes, or from about 30 minutes to about 60 minutes. In some embodiments, the composition is incubated with target material for a duration of time of less than about 45 minutes, or less than about 30 minutes, or less than about 20 minutes, or less than about 10 minutes. In some embodiments, the composition is incubated with target material for a duration of time of less than about 5 minutes.
(21) In any of the foregoing embodiments, the method can produce at least about 5% digestion of the biopolymer in the sample. In some embodiments, the method results in at least about 30% digestion of the biopolymer in the sample. In some embodiments, the method results in at least about 35% of digestion of the biopolymer in the sample. In some embodiments, the method results in at least about 40% digestion of the biopolymer in the sample. In some embodiments, the method results in at least about 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% digestion of the biopolymer in the sample. In some embodiments, the percentage of digestion is measured on a (w/w) basis. In some embodiments, the percentage of digestion is measured on a mass/mass basis.
(22) In any of the foregoing embodiments, the method can further include a step (c)(i) involving addition of an aqueous solution or water to the reaction mixture, wherein the addition of an aqueous solution or water to the reaction mixture reduces the enzymatic activity of the composition. In some embodiments, the addition of an aqueous solution or water to the reaction mixture results in changing the pH of the reaction mixture to a pH value ranging from about 4.5 to about 7.0. In some embodiments, the addition of an aqueous solution or water to the reaction mixture results in changing the temperature of the reaction mixture to a temperature ranging from about 30° C. to about 37° C.
(23) In some embodiments, the method further includes a step (c)(ii) involving adjustment of the temperature of the reaction mixture to a temperature ranging from about 30° C. to about 37° C.
(24) In some embodiments, the method further includes a step (d) of treating the reaction mixture to remove one of more contaminants.
(25) In some embodiments, treating the reaction mixture in step (d) includes removing one or more contaminants from the reaction mixture by filtration or ultra-filtration.
(26) In some embodiments, treating the reaction mixture in step (d) includes removing one or more contaminants from the reaction mixture by selective precipitation. In some embodiments, the selective precipitation is carried out by acetone precipitation, trichloroacetic acid (TCA)

precipitation, chloroform-methanol precipitation, and/or ethyl acetate precipitation. In some embodiments, the selective precipitation is carried out in deoxycholate.
(27) In some embodiments, treating the reaction mixture in step (d) includes removing one or more contaminants from the reaction mixture by chromatography. In some embodiments, the chromatography is high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC).
(28) In some embodiments, a combination of separation procedures can be used in step (d) to remove one or more contaminants from the reaction mixture, wherein the separation procedures involve one or more of filtration, ultrafiltration, selective precipitation, and chromatography.
(29) In some embodiments, the method further includes a step (e) of drying the reaction mixture.
(30) In any of the foregoing embodiments, the method can further include storing the prepared sample for a duration of time ranging from about 30 days to about 10 years. In some embodiments, the prepared sample is stored at room temperature. In some embodiments, the prepared sample is stored at 4° C. In some embodiments, the prepared sample is stored at −20° C. In some embodiments, the prepared sample is stored at −80° C.
(31) In some embodiments, the prepared sample is stored in a dried form. In some embodiments, the prepared sample is stored in dried form on a centrifugal membrane.
(32) In some embodiments, the prepared sample is stored in an aqueous form. In some embodiments, the prepared sample is stored in aqueous form in multiwell plates.
(33) In some embodiments, the prepared sample is stored in dried form in PCR tubes. In some embodiments, the prepared sample is stored in aqueous form in PCR tubes.
(34) In some embodiments, the method of any of the foregoing embodiments is part of a one-step sample preparation protocol. In some embodiments, the method is a stand-alone protocol in a multi-step sample preparation process.
(35) Also provided herein are compositions comprising enzymes that increase the efficiency, chemical ranges, substrate complexity, surfactant spectra, and speed of proteolytic digestions for mass spectrometry and other analytical applications. The operating thermal ranges of the enzymes can range from 40° C. to 110° C. at pH of 0-7. The enzymes can function in the presence of detergents or surfactants, acids, iodoacetamide (IAA), and/or dithiothreitol (DTT) among other additives.
(36) In some embodiments, the enzyme(s) included in the composition are isolated from an organism of the Archaeal domain. In some embodiments, the enzyme is isolated from an organism of the Sulfolobales order.
(37) In some embodiments, the enzyme included in the composition is selected from the group consisting of: a protease, a lipase, a cellulase, a hemicellulase, a glycoside hydrolase, an endoprotease, a carboxyesterase, an amylase, an alpha-amylase, an endoglucanase, an endopullulanase, a PNGase, a trehalase, a pullulanase, a peptidase, a signal peptidase, a xylanase, a cellobiohydrolase (CBH), a β-glucosidase, a peroxidase, a phospholipase, an esterase, a cutinase, a pectinase, a pectate lyase, a mannanase, a keratinase, a reductase, an oxidase, a phenoloxidase, a lipoxygenase, a ligninase, a tannase, a pentosanase, a malanase, a Q-glucanase, an arabinosidase, a hyaluronidase, a chondroitinase, a laccase, a xyloglucanase, a xanthanase, an acyltransferase, a galactanase, a xanthan lyase, a xylanase, an arabinase, a glycosyltransferase, a glycosidase, an endoglycosidase, an exo-glycosidase, and combinations thereof.
(38) In some embodiments, the composition further includes chemical additive as disclosed herein.
(39) In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a temperature of from about 50° C. to about 110° C. In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a temperature of from about 60° C. to about 100° C. In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a temperature of from about 70° C. to about 90° C., or from about 70° C. to about 85° C., or from about 75° C. to about 85° C., or from about 75° C. to about

80° C.
(40) In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a pH of from about 0.5 to about 7. In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a pH of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5. In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a pH of from about 4 to about 7. In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a pH of about 5.5. In some embodiments, the composition is effective for digesting biopolymers in a biological sample at a pH of about 3.0.
(41) Embodiments are also directed to a kit for digestion of a biopolymer in a biological sample, wherein the kit includes: an enzyme or enzyme mixture, an acid, optionally one or more additives, and instructions for their use, wherein the enzyme or enzyme mixture is an ultrastable, hyperthermophilic, and/or acidophilic enzyme or enzyme mixture as disclosed herein. In some embodiments, the enzyme or enzyme mixture is provided as a lyophilized product. In some embodiments, the enzyme or enzyme mixture is provided as a suspension. In some embodiments, the enzyme or enzyme mixture is provided as a solution. In some embodiments, the enzyme or enzyme mixture is immobilized on a surface.
(42) In some embodiments directed to the kit, the enzyme or enzyme mixture, the acid and the optional additive(s) are provided in separate, individual containers. In some embodiments, the enzyme (or enzyme mixture) and the acid are provided in the same container, and the optional additive(s) are provided in a separate container. In some embodiments, the acid and optional additive(s) are provided in the same container, and the enzyme (or enzyme mixture) is provided in a separate container.
(43) In some embodiments directed to the kit, the enzyme or enzyme mixture is provided in one container, and an optionally provided diluent is provided in a second, separate container. In some embodiments, instructions for preparing the enzyme or enzyme mixture in the optionally provided diluent are provided.
(44) These and other embodiments along with many of its features are described in more detail in conjunction with the text below and attached figures.

Description

BRIEF DESCRIPTION OF THE FIGURES
(1) FIG. **1** is a graph illustrating enzymatic activity of three exemplary purified and characterized ultrastable protease enzymes over a range of pH and temperature values.
(2) FIG. **2** includes protease zymograms of two purified acid-, heat-, and detergent-stable proteases using a gelatin-impregnated SDS-PAGE (1% sodium dodecyl sulfate, SDS) incubated after electrophoresis at pH 3.0 in dilute acid at 80° C. for 30 minutes.
(3) FIG. **3** includes representative Coomassie blue stained SDS-PAGE gels of BSA and Casein reactions with exemplary proteases in a log (×0.1) dilution series.
(4) FIG. **4** illustrates peptide analyses of proteolytic cleavage of casein and BSA reactions by acid- and heat-stable proteases.
(5) FIG. **5** includes a table summarizing the results of the peptide mapping and cleavage specificity of exemplified proteases described herein. CB14057 (SEQ ID NO. 26), and CB23726 (SEQ ID NO: 35) are described.
(6) FIG. **6** illustrates the compatibility of non-protease ultrastable enzymes with ultrastable proteases.
(7) FIG. **7** is a scatter plot that illustrates minimal autolysis of ultrastable proteases and protease resistance of these enzymes.

(8) FIG. **8** is a graph illustrating head-to-head comparison of the heat and acid compatibility for a single exemplary ultrastable cellulase disclosed herein compared to market leading cellulase formulation of a mix of enzymes that has been optimized for acid and heat stability.
(9) FIG. **9** is a time course graph of proteolyzed product formed using the standard tyrosine equivalence assay for two ultrastable proteses as compared to trypsin on the same bovine serum albumin (BSA) substrate

DETAILED DESCRIPTION OF THE EMBODIMENTS

(10) The methods and compositions disclosed herein generally relate to methods for exploiting the atypical characteristics of enzymes that function optimally at high temperatures and in acidic conditions. In addition, the enzymes disclosed herein retain stability and activity in a broad set of additives (e.g. detergents, surfactants, acids, and redox compounds) that render them suitable for quickly digesting biological samples in the presence of the additives for molecular analyses, including analysis by mass spectrometry (MS).
(11) Provided herein are methods for rapidly and efficiently preparing biological samples for protein analysis. The methods comprise proteolytic cleavage of biological samples using the enzymes disclosed herein to digest target proteins under conditions that promote elevated thermal and pH denaturing of target proteins, removal of post-translational modifications, and degradation of interfering molecules and structures. In some embodiments, the methods disclosed herein provide sufficient digestion to be achieved more rapidly and/or with lower enzyme doses while tolerating varied chemical reaction conditions and surfactants, leading to improved digestion and access to primary amino acid sequences in a target substrate (e.g., a three-dimensional protein with post-translational modification). Non-standard reaction conditions and additives for digestion reactions are provided during sample preparation based on the novel properties of ultrastable hyperthermophilic and/or acidophilic proteases and other enzyme classes.
(12) Previously, a suite of enzymes that function optimally extreme temperatures and highly acidic conditions was described (WO 2014/081973, incorporated herein by reference in its entirety). Disclosed herein are compositions comprising acid- and heat-stable enzymes and methods of using the same for degrading proteins and other biopolymers under extreme heat and acidic conditions in combination with detergents, surfactants and/or other chemical additives. The efficacy of combined thermal/acid/enzyme treatments for degrading proteins and other biopolymers into fragments suitable for proteomic analysis, including mass spectrometry, is demonstrated. Also provided herein are applications for degrading proteins and other biopolymers from single-celled organisms, tissues and biological fluids, using ultra-stable enzymes in combination with heat and/or acid and/or detergents and surfactants as well as other chemical additives.

1. Definitions

(13) Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art. In the event that there is a plurality of definitions for a term herein, those in this section prevail unless stated otherwise.
(14) As used herein, the singular forms “a,” “an,” and “the” include the plural reference unless the context clearly indicates otherwise. Thus, for example, reference to an “an enzyme” is a reference to one or more enzymes, etc.
(15) As used herein, the term “isolated” refers to an enzyme that is substantially or essentially free of components that normally accompany or interact with the enzyme as found in its naturally occurring environment or in its production environment, or both. Isolated enzyme preparations have less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2% or less than about 1% of contaminating protein by weight, e.g. dry weight. In some embodiments, an isolated enzyme preparation exhibits target enzyme activity of greater than 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 99.5% of detectable total enzyme activity.
(16) As used herein, the term “optimal,” in reference to enzymatic activity, refers to the ability of

the enzyme to act upon an enzyme substrate (e.g., a biomolecule) and carry out its catalytic activity, wherein the catalytic activity is the maximum activity observed at a particular parameter value relative to the activity observed over a range of parameter values the includes the particular parameter value. Parameters for assessing optimal enzymatic activity include, but are not limited to, pH, temperature, and the presence of components that can inhibit the activity of an enzyme.

(17) The term “stable” in reference to an enzyme relates to the enzyme's ability to retain its function and/or activity over time. The term “stable” is used herein as a relative term to compare the enzyme's ability to retain its function and/or activity over time in two or more different states or conditions. For example, a hyperthermophilic and/or acidophilic enzyme is referred to as being stable under high temperature and/or low pH conditions in comparison to a condition when the enzyme is not in those conditions. In some embodiments, an enzyme is stable if it retains at least about 50%, 55%, 60%, 65%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or any amount included between any two of these values, of its function and/or activity over time.

(18) The term “ultrastable” in reference to an enzyme refers to an enzyme or protein that exhibits activity at temperatures greater than about 60° C. and/or at pH values less than about 5.5. Ultrastable enzymes typically exhibit one or more “hyperthermophilic” and/or “acidophilic” traits, as discussed below, and/or tolerance for detergents, solvents, oxidizers, and other typically enzyme-incompatible chemicals at elevated temperatures and/or acidic pH. For example, in some embodiments, ultrastable enzymes exhibit stability and activity at temperatures ranging from about 60° C. to about 125° C. as described herein. In some embodiments, ultrastable enzymes exhibit activity and stability at pH values ranging from about 0.5 to about 5.5. In some embodiments, an ultrastable enzyme exhibits a half-life ranging from about 1 hour to about 300 hours at temperatures ranging from about 60° C. to about 125° C. and/or at pH values ranging from about 0.5 to about 5.5. In some embodiments, ultrastable enzymes exhibit resistance to chemical and enzymatic degradation, denaturation, and inactivation and exhibit retention of at least about 50% of enzymatic activity in the presence of a chemical and enzymatic degradant, denaturant, or inactivator relative to activity in the absence of the degradant, denaturant, or inactivator. For example, in some embodiments, ultrastable enzymes exhibit resistance to proteolysis and inactivation by mesophilic proteases and exhibit retention of at least about 50% of enzymatic activity in the presence of a mesophilic protease relative to activity in the absence of the mesophilic protease. In some embodiments, ultrastable enzymes exhibit resistance to proteolysis by hyperthermophilic proteases and exhibit retention of at least about 50% of enzymatic activity in the presence of a hyperthermophilic protease relative to activity in the absence of the hyperthermophilic protease.

(19) The term “half-life” of an enzyme typically refers to the time required for the activity of an enzyme to be reduced by one-half.

(20) The term “hyperthermophilic,” in reference to an enzyme or protein, refers to an enzyme or protein which is capable of activity at temperatures ranging from about 60° C. to about 125° C. However, in some embodiments, a hyperthermophilic enzyme or protein can operate outside of this temperature range. For example, in some embodiments, a hyperthermophilic enzyme can be active at temperatures as low as 50° C. and as high as 150° C. (i.e. encompassing the “thermophilic” range described herein). Typically, a hyperthermophilic enzyme is active at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, a hyperthermophilic enzyme exhibits at least about 10% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, a hyperthermophilic enzyme exhibits at least about 15% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included

between any two of these values. In some embodiments, a hyperthermophilic enzyme exhibits at least about 20% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, a hyperthermophilic enzyme exhibits at least about 25% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C. or at any temperature included between any two of these values. In some embodiments, a hyperthermophilic enzyme exhibits at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, "hyperthermophilic" refers to an enzyme or protein which is exhibits activity at temperatures ranging from about 65° C. to about 100° C., or from about 70° C. to about 95° C., or from about 75° C. to about 90° C., or any range included between and including any two of these values. In some embodiments, the hyperthermophilic enzyme or protein exhibits at least about 50% of its maximal activity at temperatures ranging from about 65° C. to about 100° C., or from about 70° C. to about 95° C., or from about 75° C. to about 90° C., or any range included between and including any two of these values. This is in contrast to mesophilic enzymes or components, which in general are capable of growth and/or survival, or exhibit activity, at temperatures ranging from about 20° C. to 40° C.

(21) The term "thermophilic," in reference to an enzyme or protein, refers to an enzyme or protein which is capable of activity at temperatures ranging from about 50° C. to about 150° C. Typically, a thermophilic enzyme is active at temperatures of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., 130° C., 135° C., 140° C., 145° C., 150° C., or at any temperature included between any two of these values. In some embodiments, a thermophilic enzyme exhibits at least about 10% of its maximum activity at temperatures of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., 130° C., 135° C., 140° C., 145° C., 150° C., or at any temperature included between any two of these values. In some embodiments, a thermophilic enzyme exhibits at least about 15% of its maximum activity at temperatures of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., 130° C., 135° C., 140° C., 145° C., 150° C., or at any temperature included between any two of these values. In some embodiments, a thermophilic enzyme exhibits at least about 20% of its maximum activity at temperatures of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., 130° C., 135° C., 140° C., 145° C., 150° C., or at any temperature included between any two of these values. In some embodiments, a thermophilic enzyme exhibits at least about 25% of its maximum activity at temperatures of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., 130° C., 135° C., 140° C., 145° C., 150° C., or at any temperature included between any two of these values. In some embodiments, a thermophilic enzyme exhibits at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at temperatures of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., 130° C., 135° C., 140° C., 145° C., 150° C., or at any temperature included between any two of these values. In some embodiments, "thermophilic" refers to an enzyme or protein which is exhibits activity at temperatures ranging from about 50° C. to about 100° C., or from about 55° C. to about 75° C., or from about 60° C. to about 70° C., or any range included between and including any two of these values. In some embodiments, "thermophilic" refers to an enzyme or protein which is exhibits activity at temperatures ranging from about 90° C. to about 150° C., or from about 100° C. to about

145° C., or from about 120° C. to about 140° C., or any range included between and including any two of these values. In some embodiments, the thermophilic enzyme or protein exhibits at least about 50% of its maximal activity at temperatures ranging from about 50° C. to about 100° C., or from about 55° C. to about 75° C., or from about 60° C. to about 70° C., or any range included between and including any two of these values. In some embodiments, the thermophilic enzyme or protein exhibits at least about 50% of its maximal activity at temperatures ranging from about 90° C. to about 150° C., or from about 100° C. to about 145° C., or from about 120° C. to about 140° C., or any range included between and including any two of these values.

(22) The term “acidophilic,” in reference to an enzyme or protein, refers to an an enzyme or protein that exhibits activity at pH values ranging from about 0.5 to about 5.5. However, in some embodiments, an acidophilic enzyme or protein can operate outside of this pH range, including, for example, at pH values up to about 7. Typically, an acidophilic enzyme exhibits activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. For example, in some embodiments, an acidophilic enzyme exhibits at least about 10% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, an acidophilic enzyme exhibits at least about 15% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, an acidophilic enzyme exhibits at least about 20% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, an acidophilic enzyme exhibits at least about 25% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, an acidophilic enzyme exhibits at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, “acidophilic” refers to an enzyme or protein that exhibits optimal activity at pH values ranging from about 0.5 to about 3.5, or from about 0.5 to about 2.5, or from about 0.5 to about 1.5, or from about 2.0 to about 3.0, or any range included between and including any two of these values. In some embodiments, an acidophilic enzyme or protein exhibits at least about 50% of its maximal activity at pH values ranging from about 0.5 to about 3.5, or from about 0.5 to about 2.5, or from about 0.5 to about 1.5, or from about 2.0 to about 3.0, or any range included between and including any two of these values. In some embodiments, an acidophilic enzyme or protein exhibits optimal activity or shows stability at pH values ranging from about 2.0 to about 5.0, or from about 3.0 to about 5.0, or from about 4.0 to about 5.0, or or any range included between and including any two of these values.

(23) As used herein, the terms “degrading” or “digestion,” with respect to target substrates or molecules, refers to a procedure that cleaves bonds in the target molecule to produce fragments of the original molecule. In some embodiments, the target molecule is cleaved by about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 99%, about 100%, or by any amount included between any two of these values, with respect to the total amount of target molecule on a weight or mass basis. In some embodiments, the procedure encompasses removal of post-translational modifications such as sugars, methyl groups, phosphates or other moieties that interfere with analyses as well as cleavage of the target molecule into fragments. The extent to which a target molecule is degraded or digested can be measured by any procedure known to one of ordinary skill in the art.

(24) As used herein, the terms “modifying” or “modification,” with respect to target substrates or molecules, refers to any activity that maintains the cleaved bonds in the target molecule to produce fragments of the original molecule. Exemplary modifications include, but are not limited to, reduction of disulfide bonds, methylation, acetylation, and phosphorylation. The extent to which a

target molecule is modified can be evaluated by any procedure known to one of ordinary skill in the art.

(25) As used herein, a target substrate or molecule is one that is being prepared for proteomic or other mass spectrometric analysis. The target substrate or molecule can be a biopolymer, including, but not limited to, a protein, a polypeptide, a lipid, a polysaccharide, and the like. In some embodiments, the target substrate or molecule is provided in a sample selected from the group consisting of: a residue of a grain, a dairy product, a fruit, a vegetable, a meat, an animal food, an industrial fermentation product, an algae, a biofuel, a pharmaceutical, a nutritional supplement, a tissue sample, a bodily fluid sample, a cancer biopsy, a single-celled organism, a plant, a plant part, or any combination thereof.

2. Compositions

(26) Embodiments relate to a composition useful for sample preparation and depolymerization of proteins and other biomolecules for mass spectrometry or other analytical analyses. Generally, the compositions comprise a thermally stable and/or an acid stable, and or chemically stable enzyme as disclosed herein. In some embodiments, the compositions also contain an agent useful for denaturing or degrading the biomolecule as disclosed herein. For example, the agent can be an acid, an oxidizer, a detergent, a surfactant, an additive for biopolymer digestion, a reactive and/or chaotropic chemical components, or mixtures thereof.

(27) In some embodiments, the composition has a pH value ranging from about 0.5 to about 7. In some embodiments, the compositions have a pH value ranging from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the composition has a pH of about 2.0 to 3.0. In some embodiments, the composition has a pH value ranging from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the composition has a pH of about 5.5. In some embodiments, the composition has a pH of about 3.0.

(28) Also provided herein are compositions as disclosed herein that can be applied to a sample under pH conditions ranging from about 0.5 to about 7. In some embodiments, the composition can be applied to a sample under pH conditions ranging from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the composition can be applied to a sample under pH conditions ranging from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the composition can be applied to a sample at a pH condition of about 5.5. In some embodiments, the composition can be applied to a sample at a pH condition of about 3.0.

(29) In some embodiments, the compositions disclosed herein can be employed at temperatures ranging from about 60° C. to about 125° C. For example the compositions can be applied to a sample at temperature conditions of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C. or any temperature included between any two of these values.

(30) In some embodiments, the compositions disclosed herein are heated to temperatures ranging from about 60° C. to about 125° C. prior to application to a sample. For example the compositions can be heated to a temperature of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values. Once the composition reaches its target temperature within this range, it can be employed as part of a method to degrade, digest, or otherwise prepare biological samples for analysis.

2.1. Enzymes

(31) Any enzyme or mixture of enzymes, from a source that is hyperthermophilic and/or acidophilic, can be provided in the composition, provided that the enzyme or mixture of enzymes is

stable in the desired pH range and compatible with the compositions and operating conditions disclosed herein. In some embodiments, the enzyme can be an enzyme isolated and/or produced in a manner described in WO 2014/081973, which is incorporated herein by reference in its entirety. In some embodiments, the enzyme is provided in a solid form, a liquid form, or a lyophilized form.

(32) The enzyme can be provided in an amount that is effective for sample preparation and depolymerization of proteins and other biomolecules for mass spectrometry analyses. In some embodiments, the enzyme is provided in an amount of from about 1 femtogram to 1 milligram of enzyme protein, or from about 1 nanogram to 750 micrograms (μg) of enzyme protein, or from about 1 μg to 500 μg of enzyme protein, or from about 10 μg to 250 μg of enzyme protein, or from about 25 μg to 100 μg of enzyme protein, or any amount included between any two of these values. For example, the amount of enzyme can be about 1 femtogram, 1 nanogram, 1 μg, 10 μg, 25 μg, 50 μg, 100 μg, 250 μg, 500 μg, 1 mg, or any amount included between any two of these values, of enzyme protein per 100 milligrams of sample.

(33) In some embodiments, the enzyme is provided in a concentration that ranges from about 0.0001 wt % to 50 wt %, or from about 0.001 wt % to 40 wt %, or from about 0.01 wt % to 30 wt %, or from about 0.1 wt % to 25 wt %, or from about 0.5 wt % to 20 wt %, or from about 1 wt % to 15 wt %, or from about 2.5 wt % to 10 wt %, or any range included between and including any two of these values. In some embodiments, the enzyme is provided in a concentration of about 0.0001 wt %, 0.001 wt %, 0.01 wt %, 0.1 wt %, 0.25 wt %, 0.5 wt %, 1 wt %, 2 wt %, 2.5 wt %, 3 wt %, 4 wt %, 5 wt %, 10 wt %, 15 wt %, 20 wt %, 25 wt %, 30 wt %, 35 wt %, 40 wt %, 45 wt %, 50 wt %, or any value included between any two of these values.

(34) In some embodiments, the enzyme is provided in an activity range of from about 0.0001 to 100 activity units, or from about 0.001 to 75 activity units, or from about 0.01 to 50 activity units, or from about 0.1 to 25 activity units, or from about 0.5 to 20 activity units, or from about 1 to 15 activity units, or from about 2.5 to 10 activity units, or any range included between and including any two of these values. In some embodiments, the enzyme is provided in an amount of about 0.0001 activity unit, 0.001 activity unit, 0.01 activity unit, 0.1 activity unit, 0.25 activity unit, 0.5 activity unit, 1 activity unit, 2 activity units, 2.5 activity units, 3 activity units, 4 activity units, 5 activity units, 10 activity units, 15 activity units, 20 activity units, 25 activity units, 30 activity units, 35 activity units, 40 activity units, 45 activity units, 50 activity units, 75 activity units, 100 activity units, or amount included between any two of these values.

(35) In some embodiments, the enzyme or enzyme mixture is an acidophilic enzyme or acidophilic enzyme mixture that exhibits activity at pH values ranging from about 0.5 to about 5.5. For example, in some embodiments, the acidophilic enzyme or enzyme mixture exhibits at least about 10% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, the acidophilic enzyme or enzyme mixture exhibits at least about 15% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, the acidophilic enzyme or enzyme mixture exhibits at least about 20% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, the acidophilic enzyme or enzyme mixture exhibits at least about 25% of its maximum activity at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, the acidophilic enzyme or enzyme mixture exhibits at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or at any pH value included between any two of these values. In some embodiments, the acidophilic enzyme or enzyme mixture exhibits optimal activity at pH values ranging from about 0.5 to about 3.5, or from about 0.5 to about 2.5, or from about 0.5 to about 1.5, or from about 2.0 to about 3.0, or any range included

between and including any two of these values. In some embodiments, the acidophilic enzyme or enzyme mixture exhibits at least 50% of its maximal activity at pH values ranging from about 0.5 to about 3.5, or from about 0.5 to about 2.5, or from about 0.5 to about 1.5, or from about 2.0 to about 3.0, or any range included between and including any two of these values. In some embodiments, the acidophilic enzyme or enzyme mixture exhibits optimal activity or shows stability at pH values ranging from about 2.0 to about 5.0, or from about 3.0 to about 5.0, or from about 4.0 to about 5.0, or or any range included between and including any two of these values.

(36) In some embodiments, the enzyme or enzyme mixture is stable in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture is active in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture is active in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture is stable in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture is stable at a pH of about 5.5. In some embodiments, the enzyme or enzyme mixture is stable at a pH of about 3.0.

(37) In some embodiments, the enzyme or enzyme mixture demonstrates enzymatic activity in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture demonstrates enzymatic activity in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates enzymatic activity in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture demonstrates enzymatic activity in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates enzymatic activity at a pH of about 5.5. In some embodiments, the enzyme or enzyme mixture demonstrates enzymatic activity at a pH of about 3.0.

(38) In some embodiments, the enzyme or enzyme mixture demonstrates optimal enzymatic activity in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture demonstrates optimal enzymatic activity in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates optimal enzymatic activity in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture demonstrates optimal enzymatic activity in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates optimal enzymatic activity at a pH of about 5.5. In some embodiments, the enzyme or enzyme mixture demonstrates optimal enzymatic activity at a pH of about 3.0.

(39) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 10% of its maximum enzymatic activity in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 10% of its maximum enzymatic activity in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 10% of its maximum enzymatic activity in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 10% of its maximum enzymatic activity in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 10% of its maximum enzymatic activity at a pH of

about 5.5. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 10% of its maximum enzymatic activity at a pH of about 3.0.

(40) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity at a pH of about 5.5. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity at a pH of about 3.0.

(41) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity at a pH of about 5.5. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity at a pH of about 3.0.

(42) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity at a pH of about 5.5. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity at a pH of about 3.0.

(43) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, in a pH range of from about 0.5 to about 7. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, in a pH range of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent

activity included between any two of these values, in a pH range of from about 2.0 to 3.0. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, in a pH range of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at a pH of about 5.5. In some embodiments, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at a pH of about 3.0.

(44) In some embodiments, the enzyme or enzyme mixture is a hyperthermophilic enzyme or hyperthermophilic enzyme mixture that exhibits activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, the hyperthermophilic enzyme or enzyme mixture exhibits at least about 10% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, the hyperthermophilic enzyme or enzyme mixture exhibits at least about 15% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, the hyperthermophilic enzyme or enzyme mixture exhibits at least about 20% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, the hyperthermophilic enzyme or enzyme mixture exhibits at least about 25% of its maximum activity at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, the hyperthermophilic enzyme or enzyme mixture exhibits at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or at any temperature included between any two of these values. In some embodiments, the hyperthermophilic enzyme or enzyme mixture exhibits optimal activity at temperatures ranging from about 65° C. to about 100° C., or from about 70° C. to about 95° C., or from about 75° C. to about 90° C., or any range included between and including any two of these values. In some embodiments, the hyperthermophilic enzyme or enzyme mixture exhibits at least about 50% of its maximal activity at temperatures ranging from about 65° C. to about 100° C., or from about 70° C. to about 95° C., or from about 75° C. to about 90° C., or any range included between and including any two of these values.

(45) In some embodiments, the enzyme or enzyme mixture demonstrates enzymatic activity at temperatures ranging from about 50° C. to about 125° C. For example, the enzyme or enzyme mixture demonstrates enzymatic activity at temperature conditions of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values. In some embodiments, the enzyme or enzyme mixture demonstrates optimal enzymatic activity at temperature conditions of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values.

(46) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 10% of its

maximum enzymatic activity at temperatures ranging from about 50° C. to about 125° C. For example, the enzyme or enzyme mixture demonstrates at least about 10% of its maximum enzymatic activity at temperature conditions of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values.

(47) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity at temperatures ranging from about 50° C. to about 125° C. For example, the enzyme or enzyme mixture demonstrates at least about 15% of its maximum enzymatic activity at temperature conditions of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values.

(48) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity at temperatures ranging from about 50° C. to about 125° C. For example, the enzyme or enzyme mixture demonstrates at least about 20% of its maximum enzymatic activity at temperature conditions of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values.

(49) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity at temperatures ranging from about 50° C. to about 125° C. For example, the enzyme or enzyme mixture demonstrates at least about 25% of its maximum enzymatic activity at temperature conditions of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values.

(50) In some embodiments, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at temperatures ranging from about 50° C. to about 125° C. For example, the enzyme or enzyme mixture demonstrates at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 75% of its maximum activity, or any percent activity included between any two of these values, at temperature conditions of about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values.

(51) In some embodiments, the enzyme or enzyme mixture demonstrates loss of enzymatic activity at ambient temperature and neutral pH ranges. For example, hyperthermophilic enzymes can undergo loss of activity at temperatures ranging from about 25° C. to 45° C., or from about 30° C. to 37° C. Acidophilic enzymes can undergo loss of activity at neutral pH values of from about 4.5 to 7.0 or above. In embodiments where a hyperthermophilic and/or acidophilic enzyme is provided, lowering temperature conditions to 25° C. to 45° C., and/or raising pH conditions to about 4.5 or above, can result in loss of enzymatic activity. In some embodiments, lowering temperature conditions to about 30° C. to 37° C., and/or raising pH conditions to about 4.5 to 7.0, can result in loss of enzymatic activity. In some embodiments, lowering temperature conditions to about 30° C. to 37° C., and/or raising pH conditions to about 7.0 or above, can result in loss of enzymatic activity. In some embodiments, lowering temperature conditions to about 25° C. to 45° C., or to about 30° C. to 37° C., is sufficient to result in loss of enzymatic activity. In some embodiments, raising the pH to about 4.5 or above, or to about 4.5 to 7.0, or to about 7.0 and above, is sufficient to result in loss of enzymatic activity. In some embodiments, lowering temperature conditions to about 25° C. to 45° C. and raising pH conditions to about 4.5 or above results in loss of enzymatic activity. In some embodiments, lowering temperature conditions to about 30° C. to 37° C. and raising pH conditions to about 4.5 to 7.0 results in loss of enzymatic activity. In some embodiments, lowering temperature conditions to about 30° C. to 37° C. and raising pH conditions to about 7.0 or above results in loss of enzymatic activity. Loss of enzymatic activity can mean a

reduction of at least about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of enzymatic activity relative to baseline levels at non-ambient temperatures (e.g., about 50° C. to 110° C.) and non-neutral (e.g., about 0.5 to 4.5) pH ranges.

(52) In some embodiments, the enzyme or enzyme mixture is an ultrastable enzyme or ultrastable enzyme mixture. In some embodiments, the ultrastable enzyme or enzyme mixture exhibits stability and activity at temperatures ranging from about 60° C. to about 125° C. as described herein. In some embodiments, the ultrastable enzyme or enzyme mixture exhibits activity and stability at pH values ranging from about 0.5 to about 5.5. In some embodiments, the ultrastable enzyme or enzyme mixture exhibits a half-life ranging from about 1 hour to about 300 hours at temperatures ranging from about 60° C. to about 125° C. and/or at pH values ranging from about 0.5 to about 5.5. In some embodiments, the ultrastable enzyme or enzyme mixture exhibits resistance to chemical and enzymatic degradation, denaturation, and inactivation and exhibit retention of at least about 50% of enzymatic activity in the presence of a chemical and enzymatic degradant, denaturant, or inactivator relative to activity in the absence of the degradant, denaturant, or inactivator.

(53) In some embodiments, the enzyme or enzyme mixture is a hyperthermophilic acidophilic enzyme or a hyperthermophilic acidophilic enzyme mixture. As used herein, the term “hyperthermophilic acidophilic” typically refers to an enzyme that exhibits activity (1) at temperatures ranging from about 60° C. to about 125° C., and (2) at pH values ranging from about 0.5 to about 5.5. In some embodiments, a hyperthermophilic acidophilic enzymes are active (1) at temperatures of about 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C. 125° C., or at any temperature included between any two of these values, and (2) at pH values of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.25, 4.5, 4.75, 5.0, 5.25, 5.5, or at any pH value included between any two of these values. In some embodiments, a hyperthermophilic acidophilic enzymes exhibit activity (1) at temperatures of about 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., or at any temperature included between any two of these values, and (2) at pH values of about 4.0, 4.25, 4.5, 4.75, 5.0, 5.25, 5.5, or at any pH value included between any two of these values. In some embodiments, a hyperthermophilic acidophilic enzymes exhibit at least about 50% of its maximal activity (1) at temperatures of about 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., or at any temperature included between any two of these values, and (2) at pH values of about 4.0, 4.25, 4.5, 4.75, 5.0, 5.25, 5.5, or at any pH value included between any two of these values. Hyperthermophilic acidophilic enzymes can be isolated or obtained from hyperthermophilic acidophiles or other organisms and can exhibit activity at any of the foregoing temperature and pH ranges suitable for hyperthemophilic acidophile growth and/or survival.

(54) The enzyme or enzymes provided in the composition can be a protease, a lipase, a cellulase, a hemicellulase, a glycoside hydrolase, an endoprotease, a carboxyesterase, an amylase, an alpha-amylase, an endoglucanase, an endopullulanase, a PNGase, a b-glycosidease, a trehalase, a pullulanase, a peptidase, a signal peptidase, a xylanase, a cellobiohydrolase (CBH), a β-glucosidase, a peroxidase, a phospholipase, an esterase, a cutinase, a pectinase, a pectate lyase, a mannanase, a keratinase, a reductase, an oxidase, a phenoloxidase, a lipoxygenase, a ligninase, a tannase, a pentosanase, a malanase, a β-glucanase, an arabinosidase, a hyaluronidase, a chondroitinase, a laccase, a xyloglucanase, a xanthanase, an acyltransferase, a galactanase, a xanthan lyase, a xylanase, an arabinase, a glycohydrolase, a glycosyltransferase, a glycosidase, an endo- or exo-glycosidase and combinations thereof. In some embodiments, the composition comprises a protease and a glycohydrolase. In some embodiments, the composition comprises a protease and a glycosyltransferase. In some embodiments, the composition comprises a protease and a glycohydrolase.

(55) In some embodiments, the enzyme is one that is isolated from a hyperthermophilic or thermophilic organism. In some embodiments, the enzyme is one that is isolated from an acidophilic organism. In some embodiments, the enzyme is isolated from an Archaeal organism

that is hyperthermophilic and/or acidophilic. For example, enzymes can be isolated from an organism of the Sulfolobales order, the Thermococcales order, the Thermoproteales order, the Acidilobales order, the Thermoplasmatales order, and the like. In some embodiments, the enzyme is isolated from a bacteria that is hyperthermophilic and/or acidophilic. For example, enzymes can be isolated from an organism of the Actinomycetales order, the Thermales order, the Thermoanaerobacteriales order, the Clostridiales order, the Acidothiobacillales order, the Nitrospirales order, the Rhodospirillales order, and the like. In some embodiments, the enzyme is isolated from a fungi that is hyperthermophilic and/or acidophilic.

(56) In some embodiments, the enzyme is one that can be identified and isolated as described in WO 2014/081973. Enzymes having sequences as described in WO 2014/081973 can also be suitable for use in the compositions disclosed herein. For example, protease enzymes having amino acid sequences as described in WO 2014/081973 (e.g., SEQ ID NOs: 25-35) can be incorporated into the compositions disclosed herein.

2.2. Additives

(57) At least one additive can also be employed for the compositions disclosed herein. For example, an acid may be added in order to reduce the pH to a desired pH range. Suitable acids for use in the compositions include, for example, nitric acid, phosphoric acid, hydrofluoric acid, sulfuric acid, hydrochloric acid, acetic acid, paracetic acid, peroxyacetic acid, citric acid, glycolic acid, lactic acid, formic acid, methane sulfonic acid, alkyl C.sub.8-10 polyglycolic acid, and mixtures or combinations thereof. The acid can be added in any amount ranging from about 0.1 wt % to 85 wt %, or from about 0.5 wt % to 80 wt 00 or from about 1 wt % to about 75 wt 00 or from about 2.5 wt % to about 70 wt 00 or from about 5 wt % to about 65 wt 00 or from about 10 wt % to about 60 wt 00 or from about 15 wt % to about 55 wt 00 or from about 20 wt % to about 50 wt 00 or from about 25 wt % to about 45 wt 00 or from about 30 wt % to 40 wt %, or any range included between and including any two of these values. For example, the amount of acid can be about 0.1 wt %, 0.25 wt %, 0.5 wt %, 1 wt %, 2.5, wt %, 5 wt %, 7.5 wt %, 10 wt %, 12.5 wt %, 15 wt %, 17.5 wt %, 20 wt %, 25 wt %, 30 wt %, 35 wt %, 40 wt %, 45 wt %, 50 wt %, 55 wt %, 60 wt %, 65 wt %, 70 wt %, 75 wt %, 80 wt %, 85 wt %, or any amount included between any two of these values.

(58) In some embodiments, where mixtures or combinations of two or more acids are provided, the total amount of acid can range from about 0.1 wt % to 85 wt %, or from about 0.5 wt % to 80 wt %, or from about 1 wt % to about 75 wt %, or from about 2.5 wt % to about 70 wt 00 or from about 5 wt % to about 65 wt 00 or from about 10 wt % to about 60 wt %, or from about 15 wt % to about 55 wt 00 or from about 20 wt % to about 50 wt 00 or from about 25 wt % to about 45 wt 00 or from about 30 wt % to 40 wt %, or any range included between and including any two of these values. For example, the total amount of acid can be about 0.1 wt %, 0.5 wt %, 1 wt %, 2.5, wt %, 5 wt %, 7.5 wt %, 10 wt %, 12.5 wt %, 15 wt %, 17.5 wt %, 20 wt %, 25 wt %, 30 wt %, 35 wt %, 40 wt %, 45 wt %, 50 wt %, 55 wt %, 60 wt %, 65 wt %, 70 wt %, 75 wt %, 80 wt %, 85 wt %, or any amount included between any two of these values. In an exemplary embodiment, the composition can contain about 45% nitric acid and 5% phosphoric acid.

(59) Other additives can also be provided to the composition. In some embodiments, the additives are provided to enhance biopolymer digestion. In some embodiments, the additives are provided to facilitate biopolymer modification. Exemplary additives used in proteomics and biopolymer digestion include, for example, iodoacetamide (IAA), dithiothreitol (DTT), RapiGest SF, PPS Silent® Surfactant, Invitrosol™, ProteaseMAX™, and mixtures or combinations thereof. In some embodiments, a suitable additive can be at least one selected from the group consisting of: poly(oxy-1,2-ethanediyl),alpha-(nonylphenyl)-omega-hydroxy-, dipropylene glycol monomethyl ether, sodium xylene sulfonate, potassium 4-dodecylbenzene sulfonate, triethanolamine dodecylbenzene sulfonate, triethanolamine, hydrogen peroxide, D-glucopyranose (oligomeric, decy octyl glycosides), D-glucopyranose (oligomeric, C.sub.10-6-alkyl glycosides), sodium formate,

sodium hydroxide, tetrasodium EDTA, and water.
(60) In some embodiments, the additive can comprise a solvent such as, for example, an alcohol, alkanol, polyol or a nitrile. The alkanol can be soluble or miscible with water and lipids, and comprises a C.sub.1 to C.sub.10 alkyl group that is straight or branched, substituted or non-substituted. Useful alkanols include short chain alcohols, such as C.sub.1-C.sub.8 primary, secondary and tertiary alcohols, e.g., methanol, ethanol, n-propanol, iso-propanol, and butanol. Exemplary alkanols include the various isomers of C.sub.3 alcohols, particularly iso-propanol. C.sub.1-C.sub.8 diols can also be used in the alkanol constituent. Nitrile compound such as acetonitrile can be used as the nitrile constituent in aqueous reactions.
(61) The polyol can be an alkylene glycol, such as, for example, glycerol, ethylene glycol, propylene glycol, 1,2-propylene glycol, 1,3-propylene glycol, glycerine, 1,4-butylene glycol and mixtures thereof.
(62) In some embodiments, the additive comprises an anti-foam component, such as, for example, a silicone-based anti-foam component.
(63) In some embodiments, the additive includes an alkanolamine selected from the group consisting of monoalkanolamine, dialkanolamine, trialkanolamine, alkylalkanolamine, trialkylamine, triethanolamine and combinations thereof.
(64) In some embodiments, the additive includes a conventional enzyme stabilizing agent, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, a polyamine lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester, a phenyl boronic acid derivative such as 4-formylphenyl boronic acid.
(65) In some embodiments, the additive includes a chelating agent. The chelating agent can be, for example, a metal ion chelating agent. Metal ion chelating agents can include, for example, copper, iron and/or manganese chelating agents and mixtures thereof. Such chelating agents can be selected from the group consisting of phosphonates, amino carboxylates, amino phosphonates, succinates, polyfunctionally-substituted aromatic chelating agents, 2-pyridinol-N-oxide compounds, hydroxamic acids, carboxymethyl inulins and mixtures thereof. Chelating agents can be present in the acid or salt form including alkali metal, ammonium, and substituted ammonium salts thereof, and mixtures thereof.
(66) Aminocarboxylates chelating agents include, but are not limited to, ethylenediaminetetracetates (EDTA); ethylene glycol tetraacetates (EGTA), N-(hydroxyethyl)ethylenediaminetriacetates (HEDTA); nitrilotriacetates (NTA); ethylenediamine tetraproprionates; triethylenetetraaminehexacetates, diethylenetriamine-pentaacetates (DTPA); methylglycinediacetic acid (MGDA); Glutamic acid diacetic acid (GLDA); ethanoldiglycines; triethylenetetraaminehexaacetic acid (TTHA); N-hydroxyethyliminodiacetic acid (HEIDA); dihydroxyethylglycine (DHEG); ethylenediaminetetrapropionic acid (EDTP), trans-1,2-diamino-cyclohexan-N,N,N′,N′-tetraacetic acid (CDTA), nitrilo-2,2′,2″-triacetic acid, diethylenetriamine-N,N,N′,N′,N″-pentaacetic acid, methylamine, histidine, malate and phytochelatin, hemoglobin, chlorophyll, siderophore, pyocyanin, pyoverdin, Enterobactin, peptides and sugars, humic acid, citric acid, water softeners, phosphonates, tetracycline, gadolinium, organophosphorus compound 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl, pentetic acid; N,N-Bis(2-(bis-(carboxymethyl)amino)ethyl)-glycine, N,N-bis(carboxymethyl)glycine, triglycollamic acid; [(Carboxymethyl)imino]bis-(ethylenenitrilo)]-tetraacetic acid), Trilone A, α, α′, α″-trimethylaminetricarboxylic acid, tri(carboxymethyl)amine, aminotriacetic acid, Titriplex i, and Hampshire NTA acid, and salts and derivatives thereof.
(67) Phosphorus-containing chelating agents include, but are not limited to, diethylene triamine penta (methylene phosphonic acid) (DTPMP CAS 15827-60-8); ethylene diamine tetra(methylene phosphonic acid) (EDTMP CAS 1429-50-1); 2-Phosphonobutane 1,2,4-tricarboxylic acid (Bayhibit® AM); hexamethylene diamine tetra(methylene phosphonic acid) (CAS 56744-47-9); hydroxy-ethane diphosphonic acid (HEDP CAS 2809-21-4); hydroxyethane dimethylene

phosphonic acid; 2-phosphono-1,2,4-Butanetricarboxylic acid (CAS 37971-36-1); 2-hydroxy-2-phosphono-Acetic acid (CAS 23783-26-8); Aminotri(methylenephosphonic acid) (ATMP CAS 6419-19-8); P,P′-(1,2-ethanediyl)bis-Phosphonic acid (CAS 6145-31-9); P,P′-methylenebis-Phosphonic acid (CAS 1984-15-2); Triethylenediaminetetra(methylene phosphonic acid) (CAS 28444-52-2); P-(1-hydroxy-1-methylethyl)-Phosphonic acid (CAS 4167-10-6); bis(hexamethylene triamine penta(methylenephosphonic acid)) (CAS 34690-00-1); N2,N2,N6,N6-tetrakis(phosphonomethyl)-Lysine (CAS 194933-56-7, CAS 172780-03-9), salts thereof, and mixtures thereof. Preferably, these aminophosphonates do not contain alkyl or alkenyl groups with more than about 6 carbon atoms.

(68) A biodegradable chelator that can also be used herein is ethylenediamine disuccinate (EDDS). In some embodiments, the [S,S]isomer as described in U.S. Pat. No. 4,704,233 can be used. In some embodiments, the trisodium salt of EDDA can be used, though other forms, such as magnesium salts, are also be useful. Polymeric chelating agents such as Triton P® can also be useful.

(69) Polyfunctionally-substituted aromatic chelating agents can also be used in the compositions disclosed herein. Compounds of this type in acid form are dihydroxydisulfobenzenes, such as 1,2-dihydroxy-3,5-disulfobenzene, also known as Tiron. Other sulphonated catechols may also be used. In addition to the disulfonic acid, the term “tiron” can also include mono- or di-sulfonate salts of the acid, such as, for example, the disodium sulfonate salt, which shares the same core molecular structure with the disulfonic acid.

(70) The chelating agent can also include a substituted or unsubstituted 2-pyridinol-N-oxide compound or a salt thereof, can also be provided as a chelating agent. This includes tautomers of the compound, e.g., 1-Hydroxy-2(1H)-pyridinone, as a chelating agent. In some embodiments, the chelating agent is selected from the group consisting of: 2-hydroxypyridine-1-oxide; 3-pyridinecarboxylic acid, 2-hydroxy-, 1-oxide; 6-hydroxy-3-pyridinecarboxylic acid, 1-oxide; 2-hydroxy-4-pyridinecarboxylic acid, 1-oxide; 2-pyridinecarboxylic acid, 6-hydroxy-, 1-oxide; 6-hydroxy-3-pyridinesulfonic acid, 1-oxide; and mixtures thereof. In some embodiments, the 1-Hydroxy-2(1H)-pyridinone compound is selected from the group consisting of: 1-Hydroxy-2(1H)-pyridinone (CAS 822-89-9); 1,6-dihydro-1-hydroxy-6-oxo-3-Pyridinecarboxylic acid (CAS 677763-18-7); 1,2-dihydro-1-hydroxy-2-oxo-4-Pyridinecarboxylic acid (CAS 119736-22-0); 1,6-dihydro-1-hydroxy-6-oxo-2-Pyridinecarboxylic acid (CAS 94781-89-2); 1-hydroxy-4-methyl-6-(2,4,4-trimethylpentyl)-2(1H)-Pyridinone (CAS 50650-76-5); 6-(cyclohexylmethyl)-1-hydroxy-4-methyl-2(1H)-Pyridinone (CAS 29342-10-7); 1-hydroxy-4,6-dimethyl-2(1H)-Pyridinone (CAS 29342-02-7); 1-Hydroxy-4-methyl-6-(2,4,4-trimethylpentyl)-2-pyridone monoethanolamine (CAS 68890-66-4); 1-hydroxy-6-(octyloxy)-2(1H)-Pyridinone (CAS 162912-64-3); 1-Hydroxy-4-methyl-6-cyclohexyl-2-pyridinone ethanolamine salt (CAS 41621-49-2); 1-Hydroxy-4-methyl-6-cyclohexyl-2-pyridinone (CAS 29342-05-0); 6-ethoxy-1,2-dihydro-1-hydroxy-2-oxo-4-Pyridinecarboxylic acid, methyl ester (CAS 36979-78-9); 1-hydroxy-5-nitro-2(1H)-Pyridinone (CAS 45939-70-6); and mixtures thereof.

(71) Chelating agents can also include hydroxamic acids, which are a class of chemical compounds in which a hydroxylamine is inserted into a carboxylic acid. The general structure of a hydroxamic acid is the following:

(72) ##STR00001##

(73) Suitable hydroxamates are those where R.sub.1 is C.sub.4- to C.sub.14-alkyl, including normal alkyl, saturated alkyl, salts thereof and mixtures thereof. For example, when the C.sub.8-alkyl is present, the compound is called octyl hydroxamic acid.

(74) In some embodiments, the additive can be a stabilizer, such as, for example, a hyaluronic acid stabilizer, a polyvinylpyrrolidone stabilizer, or a polyol stabilizer. Exemplary polyols are disclosed herein and include, for example, propylene glycol and glycerol. In some embodiments, the stabilizer is albumin or a sugar or sugar alcohol, such as, for example, mannitol, trehalose or

sorbitol. In some embodiments, the stabilizer is a salt, such as, for example, potassium chloride, magnesium sulfate, and the like. In some embodiments, the stabilizer is an enzyme stabilizer. Any conventional enzyme stabilizer can be used, for example, water-soluble sources of calcium and/or magnesium ions. In some embodiments, the enzyme stabilizer can be a reversible protease inhibitor, such as, for example, a lactic acid or a boron compound. Exemplary boron compounds include, but are not limited to, borate, 4-formyl phenylboronic acid, phenylboronic acid and derivatives thereof. In some embodiments, the enzyme stabilizer can be, but is not limited to, compounds such as calcium formate, sodium formate and 1,2-propane diol.

(75) The additive can be provided in the composition in any amount ranging from about 0.05 wt % to 85 wt %, or from about 0.1 wt % to 80 wt %, or from about 0.5 wt % to about 75 wt 00 or from about 1 wt % to about 70 wt 00 or from about 2.5 wt % to about 65 wt %, or from about 5 wt % to about 60 wt %, or from about 10 wt % to about 55 wt 00 or from about 15 wt % to about 50 wt 00 or from about 20 wt % to about 45 wt %, or from about 25 wt % to 40 wt %, or any range included between and including any two of these values. For example, the amount of additive provided in the composition can be about 0.05 wt %, 0.1 wt %, 0.25%, 0.5 wt %, 1 wt %, 2.5, wt %, 5 wt %, 7.5 wt %, 10 wt %, 12.5 wt %, 15 wt %, 17.5 wt %, 20 wt %, 25 wt %, 30 wt %, 35 wt %, 40 wt %, 45 wt %, 50 wt %, 55 wt %, 60 wt %, 65 wt %, 70 wt %, 75 wt %, 80 wt %, 85 wt %, or any amount included between any two of these values.

(76) The composition can include one or more surfactants, which may be an anionic surfactant, a cationic surfactant, a non-ionic surfactant, a semi-polar surfactant, a zwitterionic surfactant, a fatty acid type surfactant, a modified fatty acid surfactant, a polysorbate, an amphoteric surfactant, a polysaccharide surfactant, a silicone emulsion, a hydrotrope, or a mixture thereof.

(77) Exemplary anionic surfactants that can be provided in the compositions disclosed herein include, but are not limited to, sulfates and sulfonates, e.g., linear alkylbenzenesulfonates (LAS), isomers of LAS, branched alkylbenzenesulfonates (BABS), phenylalkanesulfonates, alpha-olefinsulfonates (AOS), olefin sulfonates, alkene sulfonates, alkane-2,3-diylbis(sulfates), hydroxyalkanesulfonates and disulfonates, alkyl sulfates (AS) such as sodium dodecyl sulfate (SDS), fatty alcohol sulfates (FAS), primary alcohol sulfates (PAS), alcohol ethersulfates (AES or AEOS or FES, also known as alcohol ethoxysulfates or fatty alcohol ether sulfates), secondary alkanesulfonates (SAS), paraffin sulfonates (PS), ester sulfonates, sulfonated fatty acid glycerol esters, alpha-sulfo fatty acid methyl esters (alpha-SFMe or SES) including methyl ester sulfonate (MES), alkyl- or alkenylsuccinic acid, dodecenyl/tetradecenyl succinic acid (DTSA), fatty acid derivatives of amino acids, diesters and monoesters of sulfo-succinic acid or soap, and combinations thereof.

(78) Exemplary cationic surfactants that can be provided in the compositions disclosed herein include, but are not limited to, alklydimethylethanolamine quat (ADMEAQ), cetyltrimethylammonium bromide (CTAB), dimethyldistearylammonium chloride (DSDMAC), and alkylbenzyldimethylammonium, alkyl quaternary ammonium compounds, alkoxylated quaternary ammonium (AQA) compounds, and combinations thereof.

(79) Exemplary non-ionic surfactants that can be provided in the compositions disclosed herein include, but are not limited to, alcohol ethoxylates (AE or AEO), alcohol propoxylates, propoxylated fatty alcohols (PFA), alkoxylated fatty acid alkyl esters, such as ethoxylated and/or propoxylated fatty acid alkyl esters, alkylphenol ethoxylates (APE), nonylphenol ethoxylates (NPE), alkylpolyglycosides (APG), alkoxylated amines, fatty acid monoethanolamides (FAM), fatty acid diethanolamides (FADA), ethoxylated fatty acid monoethanolamides (EFAM), propoxylated fatty acid monoethanolamides (PFAM), polyhydroxy alkyl fatty acid amides, or N-acyl N-alkyl derivatives of glucosamine (glucamides, GA, or fatty acid glucamide, FAGA), as well as products available under the trade names SPAN® and TWEEN®, the ethoxylates of alkyl polyethylene glycol ethers, polyalkylene glycol (e.g., 100% Breox FCC92) and alcohol alkoxylate EO/PO (e.g., Plurafac LF403). Exemplary alcohol ethoxylates include fatty alcohol ethoxylates,

e.g., tridecyl alcohol alkoxylate, ethylene oxide adduct, alkyl phenol ethoxylates, and ethoxy/propoxy block surfactants, and combinations thereof.

(80) Exemplary semipolar surfactants that can be provided in the compositions disclosed herein include, but are not limited to, amine oxides (AO) such as alkyldimethylamineoxide, N-(coco alkyl)-N,N-dimethylamine oxide and N-(tallow-alkyl)-N,N-bis(2-hydroxyethyl)amine oxide, fatty acid alkanolamides and ethoxylated fatty acid alkanolamides, and combinations thereof.

(81) Exemplary zwitterionic surfactants that can be provided in the compositions disclosed herein include, but are not limited to, betaine, alkyldimethylbetaine, sulfobetaine, and combinations thereof.

(82) Further non-limiting examples of a surfactant include a fatty acid type surfactant such as caprylic acid (e.g., 100% Prifrac 2912). Non-limiting examples of a modified fatty acid include, e.g., alkyl (C.sub.21) dibasic fatty acid, Na salt (40%, Diacid H240). Non-limiting examples of a polysorbate include potassium sorbate (e.g., Tween® 20/60/80). Non-limiting examples of an amphoteric surfactant include lauryl dimethyl betaine (e.g., Empigen BB). Non-limiting examples of a polysaccharide surfactant include alkyl C.sub.8-C.sub.10 polyglycoside (e.g., 70% Triton® BG10). Non-limiting examples of a silicone emulsion include a polydimethyl siloxane emulsion (e.g., Dow Corning Antifoam 1510).

(83) A hydrotrope is a compound that dissolves hydrophobic compounds in aqueous solutions. Typically, hydrotropes consist of a hydrophilic part and a hydrophobic part (similar to surfactants) but the hydrophobic part is generally too small to cause spontaneous self aggregation. Exemplary hydrotropes include, but are not limited to, benzene sulfonates, naphthalene sulfonates, alkyl benzene sulfonates, naphthalene sulfonates, alkyl sulfonates, alkyl sulfates, alkyl diphenyloxide disulfonates, and phosphate ester hydrotropes. Exemplary alkyl benzene sulfonates include, for example, isopropylbenzene sulfonates, xylene sulfonates, toluene sulfonates, cumene sulfonates, as well as mixtures any two or more thereof. Exemplary alkyl sulfonates include hexyl sulfonates, octyl sulfonates, and hexyl/octyl sulfonates, and mixtures of any two or more thereof.

(84) Additional exemplary surfactants include, but are not limited to, CHAPS, Big CHAP, CHAPSO, NP-40, sodium dodecyl sulfate (SDS), polysorbate 20 (Tween 20), polysorbate 80 (Tween 80), Triton® X-100, octyl glucoside, octyl thioglucoside, deoxycholate, and mixtures of combinations thereof.

(85) The surfactant can be provided in the composition in any amount ranging from about 0.05 wt % to 85 wt %, or from about 0.1 wt % to 80 wt %, or from about 0.5 wt % to about 75 wt 00 or from about 1 wt % to about 70 wt 00 or from about 2.5 wt % to about 65 wt 00 or from about 5 wt % to about 60 wt 00 or from about 10 wt % to about 55 wt 00 or from about 15 wt % to about 50 wt 00 or from about 20 wt % to about 45 wt 00 or from about 25 wt % to 40 wt %, or any range included between and including any two of these values. For example, the amount of surfactant provided in the composition can be about 0.05 wt %, 0.1 wt %, 0.25 wt %, 0.5 wt %, 1 wt %, 2.5, wt %, 5 wt %, 7.5 wt %, 10 wt %, 12.5 wt %, 15 wt %, 17.5 wt %, 20 wt %, 25 wt %, 30 wt %, 35 wt %, 40 wt %, 45 wt %, 50 wt %, 55 wt %, 60 wt %, 65 wt %, 70 wt %, 75 wt %, 80 wt %, 85 wt %, or any amount included between any two of these values.

(86) In embodiments wherein two or more surfactants are provided in the composition, the total amount of surfactant in the composition can be any amount ranging from about 0.05 wt % to 85 wt %, or from about 0.1 wt % to 80 wt %, or from about 0.5 wt % to about 75 wt %, or from about 1 wt % to about 70 wt %, or from about 2.5 wt % to about 65 wt %, or from about 5 wt % to about 60 wt %, or from about 10 wt % to about 55 wt %, or from about 15 wt % to about 50 wt %, or from about 20 wt % to about 45 wt %, or from about 25 wt % to 40 wt %, or any range included between and including any two of these values. For example, the total amount of surfactant can be about 0.05 wt %, 0.1 wt %, 0.5 wt %, 1 wt %, 2.5, wt %, 5 wt %, 7.5 wt %, 10 wt %, 12.5 wt %, 15 wt %, 17.5 wt %, 20 wt %, 25 wt %, 30 wt %, 35 wt %, 40 wt %, 45 wt %, 50 wt %, 55 wt %, 60 wt %, 65 wt %, 70 wt %, 75 wt %, 80 wt %, 85 wt %, or any amount included between any two of these

values.

3. Sample Preparation

(87) Also provided herein are methods of preparing a biological sample for analysis, wherein the method comprises: (a) providing the biological sample, (b) contacting the biological sample with a composition comprising an enzyme as disclosed herein, and (c) incubating the mixture comprising the sample and said composition for at least about one (1) second. The methods result in the digestion or modification of at least one protein or biopolymer present in the sample. Modification of the at least one protein or biopolymer present includes, but is not limited to, deglycosylation, reduction of disulfide bonds, methylation, or alkylation at one or more sites in the at least one protein or biopolymer.

(88) The biological sample can be or include any material or matter containing at least one biomolecule of interest. For example, the biological sample can be a tissue, a population of cells, a cell lysate, a cell pellet, a cell culture solution, a biological fluid (e.g., blood, milk, urine, semen), a plant tissue, a plant fluid, a food product, a gel sample, an environmental sample, a medical sample, and the like. The biological sample can be a result of a prior analytical method, such as, for example, an SDS-PAGE gel slice containing a biomolecule of interest.

(89) In some embodiments, the biological sample can be processed or treated prior to contact with the composition comprising the enzyme in step (b). For example, in embodiments wherein the biological sample is a tissue, a population of cells, or a cell culture solution, the cells in the sample can be disrupted or lysed to form a cell lysate or cell extract. Disruption of the cells can be achieved by mechanical, chemical, enzymatic and other means as are commonly known in the art. Mechanical approaches include bead beating, use of pressure such as from a French press and the like, sonication or other methods known in the art. Chemical methods include exposure to chaotropes such as urea, thiourea, or guanidine hydrochloride to lyse the cells and solubilize their contents. In some embodiments, organic acid/solvents mixtures can be utilized to disrupt cells. Enzymatic methods include using lysozyme, lysostaphin or other lytic enzymes to form “holes” in the cell walls that allow the contents to leak out into the surrounding solution. In some embodiments, a chemical or enzymatic agent is contacted with the sample prior to contacting the sample with the composition comprising the enzyme. In some embodiments, a chemical or enzymatic agent is included in the composition comprising the enzyme, and chemical or enzymatic disruption of the cells during step (c) of the sample preparation method.

(90) In some embodiments, the mixture comprising the sample and composition in step (c) is incubated at an incubation temperature that ranges from about 50° C. to about 125° C. In some embodiments, the incubation temperature is about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 110° C., 115° C., 120° C., 125° C., or any temperature included between any two of these values. In some embodiments, the incubation temperature is at least about 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., or 85° C. In some embodiments, the incubation temperature ranges from about 60° C. to about 100° C. In some embodiments, the incubation temperature ranges from about 70° C. to about 90° C. In some embodiments, the incubation temperature ranges from about 70° C. to about 85° C. In some embodiments, the incubation temperature ranges from about 75° C. to about 85° C. In some embodiments, the incubation temperature ranges from about 75° C. to about 80° C.

(91) In some embodiments, the mixture comprising the sample and composition in step (c) is incubated at a pH of from about 0.5 to about 4.5, or from about 0.5 to about 3.0, or from about 0.5 to about 1.5, or any pH range included between and including any two of these values. In some embodiments, the mixture in step (c) is incubated at a pH of about 2.0 to 3.0. In some embodiments, the mixture in step (c) is incubated at a pH of from about 4 to about 7, or from about 4.5 to about 6.5, or from about 5 to about 6, or any pH range included between and including any two of these values. In some embodiments, the mixture in step (c) is incubated at a pH of about 5.5. In some embodiments, the mixture in step (c) is incubated at a pH of about 3.0.

(92) In some embodiments, the mixture comprising the sample and composition in step (c) is incubated in the presence of an additive as disclosed herein. For example, the additive can be an acid, a protein or biopolymer digestion additive, a solvent, an anti-foam component, an enzyme stabilizing agent, a chelating agent, a stabilizer, a surfactant, a hydrotrope, and the like as described herein. In some embodiments, the additive is provided in any amount ranging from about 0.05 wt % to 85 wt %, or from about 0.1 wt % to 80 wt %, or from about 0.5 wt % to about 75 wt %, or from about 1 wt % to about 70 wt %, or from about 2.5 wt % to about 65 wt %, or from about 5 wt % to about 60 wt %, or from about 10 wt % to about 55 wt %, or from about 15 wt % to about 50 wt %, or from about 20 wt % to about 45 wt %, or from about 25 wt % to 40 wt %, or any range included between and including any two of these values. For example, the amount of additive provided in the composition can be about 0.05 wt %, 0.1 wt %, 0.25%, 0.5 wt %, 1 wt %, 2.5, wt %, 5 wt %, 7.5 wt %, 10 wt %, 12.5 wt %, 15 wt %, 17.5 wt %, 20 wt %, 25 wt %, 30 wt %, 35 wt %, 40 wt %, 45 wt %, 50 wt %, 55 wt %, 60 wt %, 65 wt %, 70 wt %, 75 wt %, 80 wt %, 85 wt %, or any amount included between any two of these values, wherein the weight percentages are based on the total weight of sample and composition. In some embodiments, the additive is provided in any amount ranging from about 0.05% (v/v) to 85% (v/v) or from about 0.1% (v/v) to 80% (v/v), or from about 0.5% (v/v) to about 75% (v/v), or from about 1% (v/v) to about 70% (v/v), or from about 2.5% (v/v) to about 65% (v/v), or from about 5% (v/v) to about 60% (v/v), or from about 10% (v/v) to about 55% (v/v), or from about 15% (v/v) to about 50% (v/v), or from about 20% (v/v) to about 45% (v/v), or from about 25% (v/v) to 40% (v/v), or any range included between and including any two of these values. For example, the amount of additive provided in the composition can be about 0.05 wt %, 0.1% (v/v), 0.25%, 0.5% (v/v), 1% (v/v), 2.5,% (v/v), 5% (v/v), 7.5% (v/v), 10% (v/v), 12.5% (v/v), 15% (v/v), 17.5% (v/v), 20% (v/v), 25% (v/v), 30% (v/v), 35% (v/v), 40% (v/v), 45% (v/v), 50% (v/v), 55% (v/v), 60% (v/v), 65% (v/v), 70% (v/v), 75% (v/v), 80% (v/v), 85% (v/v), or any amount included between any two of these values, wherein the (v/v) percentages can be based on the total volume of sample and composition.
(93) In some embodiments, the method results in at least about 5% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 10% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 15% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 20% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 25% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 30% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 35% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 40% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 45% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 50% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 55% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 60% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 65% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 70% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 75% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 80% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 85% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about 90% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in at least about

95% digestion of a protein or biopolymer in the sample. In some embodiments, the method results in about 100% digestion of a protein or biopolymer in the sample. The percent digestion can be measured on a (w/w) basis or a mass/mass basis.

(94) Incubation of the mixture comprising the sample and the composition in step (c) can be for any duration of time ranging from about 5 minutes to about 30 days. The duration of time for the incubation period can be any amount of time as along as the enzyme remains active. In some embodiments, the sample and the composition in step (c) are incubated for a duration of time ranging from about 5 minutes to about 300 minutes, or from about 10 minutes to about 150 minutes, or from about 15 minutes to about 120 minutes, or from about 20 minutes to about 90 minutes, or from about 30 minutes to about 75 minutes, or from about 40 minutes to about 60 minutes, or any range included between and including any two of these values. In some embodiments, incubation of the sample and the composition in step (c) can be for any duration of time ranging from about 1 second to about 120 minutes, or from about 30 seconds to about 100 minutes, or from about 1 minute to about 90 minutes, or from about 10 minutes to about 75 minutes, or from about 30 minutes to about 60 minutes, or any range included between and including any two of these values.

(95) In some embodiments, the mixture comprising the sample and the composition in step (c) is incubated for less than about eight hours. In some embodiments, the mixture in step (c) is incubated for less than about four hours. In some embodiments, the mixture in step (c) is incubated for less than about 120 minutes. In some embodiments, the mixture in step (c) is incubated for less than about 90 minutes. In some embodiments, the mixture in step (c) is incubated for less than about 60 minutes. In some embodiments, the mixture in step (c) is incubated for less than about 45 minutes. In some embodiments, the mixture in step (c) is incubated for less than about 30 minutes. In some embodiments, the mixture in step (c) is incubated for less than about 15 minutes. In some embodiments, the mixture in step (c) is incubated for less than about 10 minutes. In some embodiments, the mixture in step (c) is incubated for a duration of time of less than about 5 minutes. In some embodiments, the mixture in step (c) is incubated for a duration of time of less than about 1 minute. In some embodiments, the mixture in step (c) is incubated for a duration of time of less than about 30 seconds. In some embodiments, the mixture in step (c) is incubated for a duration of time of less than about 10 seconds. In some embodiments, the mixture in step (c) is incubated for a duration of time of less than about 5 seconds.

(96) In some embodiments, the mixture comprising the sample and the composition in step (c) is incubated for less than about 5 minutes. For example, the mixture in step (c) can be incubated for about 1 second, about 2 seconds, about 5 seconds, about 10 seconds, about 15 seconds, about 20 seconds, about 30 seconds, about 60 seconds, about 90 seconds, about 120 seconds, for about 3 minutes, or for about 4 minutes.

(97) In some embodiments, the mixture comprising the sample and the composition in step (c) is incubated for a duration of time ranging from about 12 hours to about 7 days, or from about 24 hours to about 6 days, or from about 36 hours to about 5 days, or from about 48 hours to about 4 days. In some embodiments, the mixture in step (c) can be incubated for a duration of time ranging from about 12 hours to about 24 hours. In some embodiments, the mixture in step (c) can be incubated for a duration of time ranging from about 24 hours to about 36 hours. In some embodiments, the mixture in step (c) can be incubated for a duration of time ranging from about 24 hours to about 48 hours. In some embodiments, the mixture in step (c) can be incubated for a duration of time ranging from about 48 hours to about 6 days. In some embodiments, the mixture in step (c) can be incubated for a duration of time ranging from about 72 hours to about 5 days.

(98) In some embodiments, the mixture comprising the sample and the composition in step (c) are incubated for a duration of time ranging from about 1 day to about 30 days, or from about 5 days to about 25 days, or from about 10 days to about 20 days. In some embodiments, the mixture in step (c) are incubated for a duration of time ranging from about 15 days to about 30 days, In some

embodiments, the mixture in step (c) can be incubated for about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 days. In some embodiments, the mixture in step (c) can be incubated for about 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 days.

(99) In some embodiments, the method further comprises a step (c)(i) of adjusting the pH of the mixture comprising the sample and the composition. In some embodiments, the pH of the mixture is adjusted to a range of about 4.5 to about 10.0. In some embodiments, the pH of the mixture is adjusted to a range of about 5.0 to about 10.0. In some embodiments, the pH of the mixture is adjusted to a range of about 7.0 to about 10.0. In some embodiments, the pH of the mixture is adjusted to about 7.5, 8.0, 8.5, 9.0, 9.5, or 10.0. The step of adjusting the pH of the mixture results in a decrease of the enzymatic activity of the composition and can be achieved by, for example, adding a sufficient amount of base, aqueous solution, or water to the mixture.

(100) In some embodiments, the method further comprises a step (c)(ii) comprising adjusting the temperature of the mixture to a temperature ranging from about 4° C. to about 37° C. In some embodiments, the temperature of the mixture is adjusted to about 4° C. In some embodiments, the temperature of the mixture is adjusted to about 10° C. In some embodiments, the temperature of the mixture is adjusted to about 12° C. In some embodiments, the temperature of the mixture is adjusted to about 15° C. In some embodiments, the temperature of the mixture is adjusted to about 20° C. In some embodiments, the temperature of the mixture is adjusted to about 25° C. In some embodiments, the temperature of the mixture is adjusted to about 30° C. In some embodiments, the temperature of the mixture is adjusted to about 32° C. In some embodiments, the temperature of the mixture is adjusted to about 35° C. In some embodiments, the temperature of the mixture is adjusted to about 37° C.

(101) In some embodiments, the step of adjusting the pH of the mixture (step (c)(i)) and/or adjusting the temperature of the mixture (step c(ii)) results in a reduction in enzymatic activity of the composition. For example, adjusting the pH of the mixture (step (c)(i)) and/or adjusting the temperature of the mixture (step c(ii)) can result in a decrease by about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of enzymatic activity relative to baseline (e.g., enzymatic activity prior to the steps (c)(i) and (c)(ii)). In some embodiments, adjusting the pH of the mixture (step (c)(i)) and/or adjusting the temperature of the mixture (step c(ii)) can result in a decrease of at least about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% of enzymatic activity relative to baseline. In some embodiments, carrying out step (c)(i) and/or (c)(ii) can result in a 100% decrease of enzymatic activity relative to baseline. In some embodiments, carrying out step (c)(i) and/or (c)(ii) can result in complete elimination of enzymatic activity relative to baseline. In some embodiments, step (c)(i) is carried out to reduce enzymatic activity as disclosed herein. In some embodiments, step (c)(ii) is carried out to reduce enzymatic activity as disclosed herein. In some embodiments, steps (c)(i) and (c)(ii) are carried out to reduce enzymatic activity as disclosed herein.

(102) In some embodiments, steps (a) to (c) (including (c)(i) and (c)(ii), if undertaken), are carried out in a single vessel or container.

(103) In some embodiments, subsequent to step (c), (c)(i), and/or (c)(ii), the mixture is further treated to remove contaminants. In some embodiments, subsequent to step (c), (c)(i), and/or (c)(ii), the mixture, or a portion thereof, is directly analyzed for proteomic, glycomic, glycoproteomic analysis. For example, in some embodiments, subsequent to step (c), (c)(i), and/or (c)(ii), the mixture, or a portion thereof, is injected into a mass spectrometer device for analysis.

(104) In some embodiments, subsequent to step (c), (c)(i), and/or (c)(ii), the mixture undergoes a treatment step (d) that allows removal of contaminants and sample clean-up for subsequent analysis by mass spectrometry. The treatment step (d) results in removal of salts or lipids from the crude cell lysate or extract, removal of organic solvents and/or chemical additives in in the mixture, and enrichment of one or more analytes of interest (e.g. a digested protein or biopolymer) relative to

one or more other components of the sample.
(105) In some embodiments, treatment of the mixture in step (d) comprises removing one or more contaminants by filtration of ultrafiltration. Filtration and ultrafiltration techniques are known to those of skill in the art, e.g., as described by Ivanov and Lazarev (2011. *Sample preparation in biological mass spectrometry.* Dordrecht: Springer, xxix, 1089 pages).
(106) In some embodiments, treatment of the mixture in step (d) comprises removing one or more contaminants by selective precipitation. In some embodiments, the selective precipitation is carried out by acetone precipitation, trichloroacetic acid (TCA) precipitation, chloroform-methanol precipitation, and/or ethyl acetate precipitation. Selective precipitation techniques are known in the art, and can be carried out in accordance with protocols described, for example, in Ivanov and Lazarev (2011. *Sample preparation in biological mass spectrometry.* Dordrecht: Springer, xxix, 1089 pages).
(107) In some embodiments, treatment of the mixture in step (d) comprises removing one or more contaminants by chromatography. Chromatographic separation methods include one or more of ion exchange, size exclusion, hydrophobic liquid interaction chromatography (HILIC), hydrophobic interaction, affinity, normal-phase, or reverse-phase chromatography. In some embodiments, chromatography is carried out using a chromatography column that is configured for at least partial chromatographic separation and isolation of the digested proteins or biopolymer in the sample. The stationary phase in the chromatography column can be porous or non-porous silica or agarose particles, or a monolithic material polymerized or otherwise formed inside the column. The stationary phase can be coated with an appropriate material such as C18, C8, C4 or another suitable derivative, or contain cation exchanger or other material, or the combination of the above to facilitate the separation of the proteins, and such material may be chemically bonded to the particles or monolith inside the column. Particle sizes typically range from about 1.5 μm to 30 μm. Pore sizes can range from 50 to 300 angstroms. Inside diameters of columns typically range from about 50 μm to 2.1 mm, and column length from about 0.5 cm to 25 cm or longer. In some embodiments, the mobile phase or eluent can be a pure solvent, or a mixture of two or more solvents, and may contain added salts, acids and/or other chemical modifiers. In some embodiments, the proteins are separated on the column based on one or more physiochemical properties, including size, net charge, hydrophobicity, affinity, or other physiochemical properties. In some embodiments, the chromatography technique comprises high-performance liquid chromatography (HPLC). In some embodiments, the chromatography process comprises ultra-performance liquid chromatography (UPLC). Chromatography, HPLC, and UPLC techniques are known in the art and are described, for example, in Ivanov and Lazarev (2011. Sample preparation in biological mass spectrometry. Dordrecht: Springer, xxix, 1089 pages).
(108) In some embodiments, treatment of the mixture in step (d) comprises removing one or more contaminants by a sample-purification device, such as, for example, a solid phase extraction (SPE) cartridge. In some embodiments, the SPE cartridge is in line directly with the high resolution/accurate mass instrument. In some embodiment, the SPE cartridge is a polypropylene tip with a small volume of silica or other sorbent containing bonded C4, C8, C18, RP4H, or RPSH or other functional groups immobilized in the cartridge, for example, a StageTip™ cartridge (Thermo Fisher Scientific). In some embodiments, polymeric sorbents or chelating agents are used. The bed volume can be as small as 1 μL or less but greater volumes are also contemplated. In some embodiments, the SPE cartridge is used once.
(109) In some embodiments, treatment of the mixture in step (d) can include one or more of the techniques described supra. For example, in some embodiments, the treatment step (d) can comprise a filtration step and a selective precipitation step. In some embodiments, the treatment step (d) can comprise a filtration step and a chromatography step. In some embodiments, the treatment step (d) can comprise a selective precipitation step and a chromatography step. In some embodiments, the treatment step (d) can comprise a filtration step, a selective precipitation step,

and a chromatography step. The filtration step, selective precipitation step, and chromatography step can be carried out in any sequence order. Treatment of the mixture in step (d) typically results in sufficient removal of one or more contaminants such that digested protein or biopolymer in the prepared sample is suitable for analysis, e.g., by mass spectrometry. For example, treatment of the mixture in step (d) can provide sufficient removal of one or more contaminants such that the one or more contaminants is undetectable or provides minimal interference during analysis of the sample.

(110) In some embodiments, the method further comprises a step (e) of drying the mixture. In some embodiments, step (e) results in removal of about 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 99.5% of the liquid in the mixture. Drying the mixture can be carried out by placing the sample at an elevated temperature (≥37° C.) and/or under vacuum. In some embodiments, drying the mixture can be carried out by lyophilization.

(111) In some embodiments, subsequent to any of steps (c) (e.g., subsequent to step (c), step (c)(i), step (c)(ii)), step (d), and/or step (e)), the method further comprises storing the mixture containing the prepared sample for a duration of time ranging from about 30 days to about 10 years. In some embodiments, the mixture is stored for at least about 30 days. In some embodiments, the mixture is stored for at least about 45 days. In some embodiments, the mixture is stored for at least about 60 days. In some embodiments, the mixture is stored for at least about 90 days. In some embodiments, the mixture is stored for at least about six months. In some embodiments, the mixture is stored for at least about a year. Storage conditions include temperatures ranging from about −70° C. to room temperature (approximately 25° C. to 28° C.).

4. Common Applications

(112) The compositions and methods of using the same for sample preparation as disclosed herein can be applied to any type of analytical method, including, but not limited to mass spectrometry-based proteomic analysis, glycomic analysis, glycoproteomic analysis, lipomic analysis, amino acid analysis, enzymatic assay, and immunochemical assay, among other biological and biochemical analyses.

(113) In some embodiments, the compositions and methods of sample preparation disclosed herein are directed to use in mass spectometry based proteomics. There are two main approaches for mass spectrometry-based proteomics, top-down and bottom-up analyses. Top-down methods analyze whole proteins, while bottom-up approaches investigate the peptides from digested proteins. The compositions and methods disclosed herein have broad applicability to bottom-up approaches for analysis but are not limited to bottom-up approaches. In some embodiments, the compositions and methods disclosed herein can be used in further evaluation of a biological sample after top-down analysis has taken place. The sample is contacted with a composition comprising

(114) In some embodiments, the compositions and methods of sample preparation disclosed herein are directed to use in immunochemical analysis. In some embodiments, a composition comprising a protease is contacted with the sample for analysis by immunochemistry. In some embodiments, a composition comprising a protease and a glycohydrolase is contacted with the sample for analysis by immunohistochemistry.

5. Kits

(115) Also provided herein are kits for preparing or digesting analytical samples, wherein the kit comprises: an enzyme or enzyme mixture, an acid, optionally one or more additives, and instructions for their use. The enzyme or enzyme mixture can be a thermophilic, hyperthermophilic and/or acidophilic enzyme as described herein. The acid and optional additive can be any acid and additive as disclosed herein.

(116) In some embodiments, the enzyme or enzyme mixture is provided as a lyophilized product, which can optionally be provided with a diluent. In some embodiments, the enzyme or enzyme mixture is provided as a suspension. In some embodiments, the enzyme or enzyme mixture is provided as a solution. In some embodiments, the enzyme or enzyme mixture is provided in one container, and the optionally provided diluent is provided in a second, separate container. In some

embodiments, instructions for preparing the enzyme or enzyme mixture in the optionally provided diluent are provided.

(117) In some embodiments, the enzyme or enzyme mixture, the acid and the optional additive(s) are provided in separate, individual containers. In some embodiments, the enzyme (or enzyme mixture) and the acid are provided in the same container, and the optional additive(s) are provided in a separate container. In some embodiments, the acid and optional additive(s) are provided in the same container, and the enzyme (or enzyme mixture) is provided in a separate container.

(118) In some embodiments, the kit comprises a microfluidics apparatus, and the enzyme or enzyme mixture is immobilized on a structure that forms part of the apparatus. In such embodiments, a sample can be provided to the apparatus and digested, cleaved, or otherwise prepared for analysis during in-line flow as part of the upstream fluidics of an analyzer, e.g., a mass spectrometer.

(119) In some embodiments, the kit comprises an enzyme mixture comprising an ultrastable enzyme and at least one mesophilic enzyme. Temporally-distinct digestions of the sample can be carried out by sequentially incubating the enzyme mixture and sample at a first temperature at which the at least one mesophilic enzyme is optimally active, followed by incubation at a second temperature at which the ultrastable enzyme is optimally active, optionally followed by incubation at one or more sequential temperatures in which each sequential temperature corresponds to a temperature at which one or more additional ultrastable enzymes is optimally active. Such “thermal switching” allows multiple sequential activities to be applied to a single sample separated by time using a single formulation and segmented temperature incubations to control the respective activities.

(120) In some embodiments, the kits can be stored at ambient (about 20° C.-25° C.) temperatures. In some embodiments, the kits can be stored at about 4° C. In some embodiments, the kits can be stored at temperatures of from about 4° C. to about 20° C. In some embodiments, the kits can be stored at temperatures of up to about 30° C.

(121) In some embodiments, the kits have a storage shelf-life of at least about three months. In some embodiments, the kits have a storage shelf-life of at least about six months. In some embodiments, the kits have a storage shelf-life of at least about nine months. In some embodiments, the kits have a storage shelf-life of at least about 12 months, 18 months, 24 months, 30 months or 3 years.

EXAMPLES

Example 1

(122) Production of Candidate Ultrastable Enzymes

(123) Potentially useful gene sequences were identified using standard bio-informatics approaches. Genes of interest were isolated and cloned using standard molecular biology techniques according to a scheme similar to those disclosed in WO 2014/081973, which is incorporated herein by reference in its entirety. Functional enzymes were produced by recombinant expression in hyperthermophilic and acidophilic microbes of the domain Archaea of the order Sulfolobales. Transformed microbes were cultured at 80° C. and pH=3.0, and culture medium included carbon, nitrogen, phosphorous, and sulfur sources and trace minerals. Genetic constructs of genes of interest were designed to target gene products to the extracellular space using localization sequences similar to those described previously (WO 2014/081973). Recombinant enzymes accumulated in the culture media and were concentrated and buffer exchanged using commercially available tangential flow filtration devices. In some embodiments, enzymes were designed to have an epitope, a poly-histidine fusion (e.g., a histidine tag) or another useful modification to facilitate purification and/or characterization. Enzymes were concentrated 200-10,000× from the original solution and filter sterilized and stored at room temperature, −20° C., −80° C. or lyophilized. Further chromatographic purifications are carried out for each individual enzyme to >99% homogeneity for the activity of interest.

(124) Enzymes suitable for acidic pH environments have at least 25% of their maximum activity at pH values ranging from about 0.5 to 4.5. Exemplary optimum activities range from about pH 2.5 to 3.5. Enzymes suitable in neutral pH environments have at least 25% of their maximum activity pH values ranging from about 4 to 7. Exemplary optimum activity for such an enzyme can be at about pH 5.5.
(125) Enzymes suitable for hyperthermophilic environments have at least 25% of their maximum activity at temperatures ranging from about 70° C. to about 110° C. Exemplary optimum activities range can be from about 70° C. to about 90° C., or from about 75° C. to about 85° C., or at about 80° C.
Example 2
(126) Characterization of Ultrastable Protease Enzymes
(127) Three exemplary proteases were purified and assayed for enzymatic activity over a range of pH and temperature values. Enzymatic activity was assayed by standard protease assay holding one parameter at a fixed value while varying the values of the other parameter. Operational ranges were defined by ≥50% maximal activity. Approximate optimal temperatures, pH, and half-life were measured and are indicated in FIG. **1**.
(128) Two of the proteases were further analyzed for detergent, acid, and thermal stability by assessing different mobility patterns on a gelatin-impregnated SDS-PAGE (1% SDS). The SDS-PAGE gel was incubated after electrophoresis at pH 3.0 in dilute acid at 80° C. for 30 minutes. As illustrated in FIG. **2**, protease activity is visible as a white bands or smears against the blue background, which indicates digestion of gelatin protein impregnated throughout the gel matrix. FIG. **2** thus illustrates retained protease activity at acidic pH (3.0) and elevated temperature (80° C.) after exposure to SDS during the running of the gel, for the assayed protease enzymes, indicating detergent, acid, and thermal stability of the enzymes.
Example 3
(129) Comparison of Exemplary Enzymes to Commercial Formulations
(130) The enzymes disclosed herein were compared to commercially available formulations (e.g., Novozymes CTec2) that were optimized for acid and heat stability over many years. In the comparison study, the enzymes described herein significantly outperformed the commercial formulations (FIG. **8**). For example, it was observed that the ultrastable cellulase optimal activity occurs at a temperature that is 30° C. higher than that of comparable commercially available cellulase enzymes. In addition, it was observed that the ultrastable cellulase functioned optimally in a pH range that is >300× more acidic (by about 2.5 pH units) than the functional pH range of commercially available comparators.
Example 4
(131) Evaluation of Proteases for Sample Preparation (Ms-Based Proteomic Analysis)
(132) Experiments were conducted to apply hyper-heat and acid stable proteases to in-solution digestions of common proteins for proteomic analysis. Due to the large amount of available proteomics data for BSA, casein, myoglobin and ovalbumin, preliminary proteomics analyses of digestion of BSA and casein with three candidate proteases were carried out (FIG. **3**). Each protease enzyme was incubated with BSA or casein for 1 hour at pH 3.0 in dilute acid at 80° C. A representative reaction was selected form each series, and the products were analyzed by tandem mass spectrometry to identify the resulting peptides. FIG. **4** illustrates the peptide analysis of proteolytic cleavage of casein and BSA by the proteases. Approximately 1000 peptide ions were scored for each digestion to preliminarily map the cleavage pattern of the respective proteases on BSA and casein. The number of peptide ions identified with the indicated amino acids at the P1 and P1′ locations are indicated in FIG. **4**. FIG. **5** includes a table summarizing the results of the peptide mapping and cleavage specificity of the tested proteases.
(133) The results suggested that one candidate protease is pepsin-like. In contrast, the other two candidate proteases showed novel cleavage specificity (not all data shown). Additional research is

needed to further characterize the candidate proteases, including identification of key parameters for in-solution digests including; coverage statistics, cleavage specificity, and signal intensities (digestion efficiencies) and benchmarking against commercial trypsin protocols for mass spectrometry.

Example 5

(134) Compatibility of Non-Protease Ultrastable Enzymes with Ultrastable Protease Enzymes

(135) Non-protease ultrastable enzymes were incubated with ultrastable protease enzymes to determine sensitivity of the non-protease ultrastable enzymes to degradation by the ultrastable protease enzymes.

(136) In a first experiment, unique combinations of an ultrastable lipase enzyme mixed with an ultrastable protease enzyme were spotted on a gellan gum plate containing a biological fat in the form of a triacylglycerol (tributyrin) emulsion with a pH of 3.0. The plate was then incubated at 80° C. for 60 minutes. As illustrated in FIG. **6** (top panel), three of the protease/lipase combinations illustrated lipase sensitivity to degradation by the protease, while one combination illustrated lipase stability in the presence of protease (top row, third column).

(137) In a second experiment, an ultrastable amylase enzyme was incubated in the presence or absence of one of two ultrastable proteases, and activity of the amylase enzyme was monitored for one hour at the optimal conditions for each protease. Activity was measured in triplicate with a standard biochemical assay for amylase activity. As illustrated in FIG. **6** (bottom panel), the amylase enzyme retained its activity in the presence of both tested proteases.

(138) In a third experiment, two ultrastable proteases were incubated together, and cross-compatibility activity of the proteases was assayed. The proteases were incubated separately or together at 80° C., pH 3.0 for up to five days and subsequently assayed using a standard biochemical assay for protease activity. As illustrated in FIG. **7**, the results indicated that over 50% of original activity was exhibited by the mixture of proteases under the tested conditions, indicating resistance to proteolysis for both enzymes.

(139) The results indicate that ultrastable lipase/protease, amylase/protease, and protease/protease combinations can be used on enzyme substrates without incurring enzyme inactivation by protease activity.

Example 6

(140) Optimization of Enzyme Concentration (Ms-Based Proteomic Analysis)

(141) A series of enzyme/substrate ratios was tested at the defined optima for the candidate (“CB”) proteases. Initially, the assays were carried out for one hour and visualized on coomassie brilliant blue (CBB)-stained SDS-PAGE as described (Example 4, FIG. **3**). The initial experiment was used to approximately define appropriate enzyme concentrations for digestion reactions for various substrates and to guide serial enzyme concentration amounts for the proteomic analysis with finer gradation between concentration points. Initial rates were estimated from the linear regression of initial points before the break from linearity. All reactions were prepared with 1 μg of tested enzyme and 200 μg of BSA substrate, and enzyme/substrate mixtures were incubated at the temperature and pH optima for each tested enzyme. As enzyme concentration depends on time and substrate concentration, biochemical experimental data was also collected at various enzyme concentrations to provide information on enzyme amounts in formulations (FIG. **9**). The results illustrated in FIG. **9** indicate that the tested ultrastable proteases exhibited significantly higher digestion rates of BSA compared to trypsin under their respective optimal conditions.

Example 7

(142) Optimization of Sample Incubation (Digestion) Times (MS-Based Proteomic Analysis)

(143) For each candidate enzyme, after defining the enzyme to substrate ratios best suited for proteomic analyses, the relative effectiveness of a selected enzyme concentration with various substrates is examined and compared to results using higher enzyme concentrations and shorter incubation times and/or lower enzyme concentrations and longer incubation times. Since adequate

digestion with the benchmark enzyme trypsin typically requires 4 to 24 hours, the focus is on identifying reaction conditions that provide maximum digestion and coverage in less than 60 minutes. Based on preliminary experiments in which candidate proteases were compared to trypsin, there is a potential for significantly reduced digestion times (˜ 1/10×) relative to trypsin (FIG. **9**). Based on the preliminary experiments, it was observed that trypsin did not break from linearity while the candidate ("CB") enzymes digested enough BSA to break from linearity (i.e. sub-saturating substrate concentrations). Further studies are needed to investigate enzyme amount and reaction condition variables with readouts to include mass spectrometry in addition to biochemical assays.

Example 8

(144) Optimization of Sample pH (MS-Based Proteomic Analysis)

(145) Experiments to quantify the level and specificity of chemical hydrolysis from the heated acid reaction conditions for candidate enzymes on a set of test proteins are carried out. Previously, it was determined that candidate ("CB") protease enzymes exhibited nearly equivalent levels of biochemical activity in nitric, phosphoric, sulfuric, and citric acids (data not shown). Since these acids may have differing background hydrolysis or amino acid side chain chemistries at elevated temperatures, activity and acid hydrolysis of candidate enzymes in the presence of various acids set across a range of pH (1.5-4 at 80° C.) is carried out. In circumstances, certain pH can cause precipitation of target protein. Accordingly, experiments are carried out to compare the proteolytic performance under target protein precipitating pH conditions relative to other pH values that show less, or no, precipitation of target protein along the tested pH gradients. The results of such studies provides a basis for acids and pH values that are useful for formulating reaction mixes for commercial proteomics products.

Example 9

(146) Optimization of Sample Temperature (MS-Based Proteomic Analysis)

(147) As considerable efforts are being put towards automation of proteomic samples, including front-end immobilized enzyme reactor (IMER) technologies, candidate enzymes are investigated for use in automated processes involving enzyme immobilization and re-use for proteomics. To identify a practical intersect between temperatures and proteolytic performance, a study to investigate the function of candidate enzymes (e.g. proteases) in the context of proteomic mass spectrometry at temperatures below and above an identified optima is carried out. Lower temperatures may provide gains in enzyme half-lives for IMER and other relevant contexts. In contrast, elevated temperatures may reduce reaction times for one-off digestion applications.

Example 10

(148) Assessment of Candidate Enzymes for Glycoproteomic Applications

(149) Candidate enzymes are investigated for potential activity in debranching or depolymerizing glycans or cleave O- and N-linked sugar/protein bonds. Posttranslational modifications, particularly large and heterogeneous glycosylations, can interfere with proteases, chromatography, and yield limited protein coverage. Glycosylation of a large fraction of target proteins is particularly pronounced in membrane proteomics and neurobiology among other fields. Mesophilic enzymes are currently a leading option for the removal and/or degradation of these complex sugars for mass spectrometry analyses. However, the currently available enzymes require separate steps prior to proteolytic reactions, as trypsin degrades and inactivates the glycan-acting enzymes if the two enzymes are incubated together. The objective of such studies is to identify hyperstable candidate enzymes that can positively impact proteomic analysis of glycoproteins in addition to retaining their enzymatic activity despite the heat and acid of the reaction conditions. In a more particular embodiment, isolated thermo-acid stable glycohydrolases are investigated for their thermostability, utility in glycoprotein proteomics, and compatibility with candidate proteases.

(150) Test substrates can include, e.g., RNase B, for demonstration of N-linked deglycosylation using SDS-PAGE and proteomics, and interleukin-6 and α1-Acid Glycoprotein for O- and N-linked

deglycosylation (Sigma). Candidate enzymes are tested for activity on these glycoprotein substrates. Experimental readouts include, e.g., gel mobility alteration and changes in protein coverage using proteomic data. Biochemical assays for detecting free sugars are also used if appropriate. Positive controls include commercially available protein deglycosylation kits (Sigma).
(151) A matrix of each result generated from reaction between a candidate enzyme and a substrate, as evaluated by SDS-PAGE stained with coomassie brilliant blue (CBB) or Schiff stain for glycans is produced. The initial experiments provide a coarse readout on the enzymes that have the most significant effect on the glycosylation, and the class(es) of glycans that are acted upon by using the various substrates. Collation of these data is used to guide follow-on experiments. Once a set of promising candidate enzymes is determined, proteomic analyses is carried out with trypsin (benchmark) and the candidate proteases using reaction conditions determined prior (Examples 3-7). Identification of enzymes that deglycosylate the substrates in a manner that liberates peptides from the linked glycans is carried out based on these data. The utility of such hyperstable glycan enzymes for single-step glycoproteomic reactions is further assessed and formulated with hyperstable candidate proteases.

Example 11

(152) Assessment of Compatibility of Ultrastable Proteases with Glycohydrolases for Formulation
(153) Commercially available deglycosylation kits and procedures involve many steps and are laborious and time consuming (e.g., Sigma deglycosylation-kits). Glycoproteomic protocols are generally multiple step, can be somewhat complex, time consuming, and require extensive sample handling and subsequent losses and introduce significant sample-to-sample variation. Much of the process time and steps can be attributed to sequential incubations and drying to remove SDS from gel slices and to compensate for the incompatibility of glycan enzymes and protease enzymes. Some protocols also involve a thermal denaturation step of 100° C. to assist in downstream enzymatic digestion. Accordingly, there are potential advantages for combining multiple steps into a single process step that significantly reduces time, handling, and variability. Specifically, heat denaturation, deglycosylation, and proteolytic digestion that are tolerant of SDS from gel slices can be carried out in a single step using ultrastable enzyme formulations.
(154) A limited set of pilot experiments was carried out to 1) further assess a library of potentially useful glycan enzymes, and 2) assess the compatibility of these enzymes with proteases for acid/heat/detergent stable formulations. Guided by the results from Example 9, the relative stability of glycan-cleaving enzymes in the presence of hyperstable proteases was assessed. Pilot experiments for two candidate glycohydrolases were previously carried out, and both enzymes retained >95% of their activity after one hour at 80° C. and pH=3 in the presence of excess of two candidate proteases (data not shown).
(155) However, not all of candidate enzymes are resistant to protease cleavage, suggesting that 1) various enzymes have differing resistance to hyperstable candidate proteases, and 2) the resistance can be pairwise-specific. To illustrate these points, a matrix of pH, protease, and lipase reactions with a visual readout assay is provided in FIG. **6**. In FIG. **6**, the results were generated by reacting two different lipases with two different proteases with pH 3 or 6 buffer controls for 30 minutes at 80° C. Aliquots of 7.5 μL of the resulting reaction mixtures were then spotted onto a solid matrix plate with a pre-formed emulsion of ghee (clarified butter) at pH=3 and incubated at 80° C. for 30 minutes and photographed against a dark background. The lipase activity on the ghee emulsion is visualized as clearing of opacity, to notably differing degrees for the different lipases (FIG. **6**, top two rows). In particular, this experiment revealed a combination of protease and lipase that were compatible for co-formulation (FIG. **6**, box), while other protease/lipase combinations resulted in significantly diminished lipase activity. These data indicate that not all hyperstable candidate enzymes are equally resistant to various hyperstable candidate proteases.
(156) A similar matrix of tests between the proteases and glycan-digesting enzymes is executed. Protease compatibility of glycohydrolases is assessed by established biochemical assays for each

relevant glycohydrolase activity being tested as compared to mock reactions lacking protease. Remaining glycohydrolytic activity after protease pre-treatment indicates protease resistance, and the values give an indication of the level of resistance and allow ranking of candidate glycohydrolases for co-formulation with candidate proteases.

Example 12

(157) Assessing Simultaneous Deglycosylation and Proteolysis of Candidate Enzymes

(158) Glycohydrolases that show potential for removing glycans to allow identification of modified peptides are further tested. A subset of enzymes that show incompatibility with candidate proteases is tested in a two-step deglycosylation protocol. The protease-tolerant glycohydrolases (as identified) are tested for their impact on proteomic coverage of the glycoprotein substrates in single-step glycoproteomic reactions. Initial incubation times, enzyme doses, and optimal pH and temperatures for reactions is guided by previous experimentation (e.g., as illustrated in Example 2) as well as historical data. The objective of these experiments is furthering the development of a set of products that simplify glycoproteomic and proteomic sample preparation. These products can take many forms, however, experimentation to date encourages an embodiment of a dried 96-well plate format that is stable at room temperature and requires only rehydration, sample addition, and incubation prior to proteomic analysis.

Example 13

(159) Preparation of a Sample Using an Enzyme Mixture

(160) A biological sample is obtained and incubated with a composition containing a mesophilic glycohydrolase and an ultrastable protease. The mixture is incubated at 37° C. for one hour and subsequently incubated at 80° C. for one hour. The mixture is optionally incubated at a pH of between 2 to 5 for one or both incubation periods. Incubation at the lower temperature allows enzymatic cleavage of carbohydrates at glycosylated sites in proteins of the sample. Subsequent incubation at the higher temperature allows enzymatic digestion of the proteins in the sample to produce smaller peptide fragments for proteomic analysis. After the second incubation period, the sample is injected onto a mass spectrometer for proteomic analysis.

Example 14

(161) Preparation of a Sample for Lipomic Analysis

(162) A biological sample is obtained and incubated with a composition an ultrastable lipase and optionally, an ultrastable protease. The mixture is incubated at 80° C. for one hour. The mixture is optionally incubated at a pH of between 2 to 5 and/or optionally incubated in the presence of a detergent, a surfactant, and/or a redox compound. After the incubation period, the sample is analysed for lipomic analysis.

Example 15

(163) Preparation of a Sample for Glycomic Analysis

(164) A biological sample is obtained and incubated with a composition an ultrastable amylase and optionally, an ultrastable protease. The mixture is incubated at 80° C. for one hour. The mixture is optionally incubated at a pH of between 2 to 5 and/or optionally incubated in the presence of a detergent, a surfactant, and/or a redox compound. After the incubation period, the sample is analysed for glycomic analysis.

(165) One or more features from any embodiments described herein or in the figures may be combined with one or more features of any other embodiments described herein or in the figures without departing from the scope of the invention.

(166) All publications, patents and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without

departing from the spirit or scope of the appended claims.

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Claims

1. A method of preparing a biological sample, comprising: (a) providing the biological sample comprising at least one biopolymer; (b) contacting the sample with a composition comprising an ultrastable enzyme from an organism of the Archaea domain, to form a reaction mixture, wherein the ultrastable enzyme cleaves the biopolymer at one or more specific sites, and wherein the ultrastable enzyme is selected from the group consisting of SEQ ID NO: 26 and SEQ ID NO: 35; and (c) incubating the reaction mixture for at least one second to digest the at least one biopolymer present in the biological sample, at a pH between 0.5-7.0 and a temperature between 50° C.-150° C., and wherein steps (a) to (c) produce a prepared sample for proteomic, glycomic, or glycoproteomic analysis, and wherein the prepared sample is injected into an analytical device for proteomic, glycomic, or glycoproteomic analysis after step (c).

2. The method of claim 1, wherein the biological sample is prepared for mass spectrometry-based proteomic, glycomic, glycoproteomic, lipomic, amino acid, enzymatic, or immunochemical analysis.

3. The method of claim 1, wherein the sample is selected from the group consisting of a tissue, a cell pellet, a cell lysate, a cell culture solution, a biological fluid, a food product, and a gel sample.

4. The method of claim 1, wherein the composition of step (b) further comprises an acid.

5. The method of claim 4, wherein the composition of step (b) comprises an acid, wherein the acid is selected from the group consisting of nitric acid, phosphoric acid, hydrofluoric acid, sulfuric acid, hydrochloric acid, acetic acid, paracetic acid, citric acid, glycolic acid, formic acid, and combinations thereof.

6. The method of claim 1, wherein the composition of step (b) further comprises a surfactant or detergent.

7. The method of claim 1, wherein the composition of step (b) further comprises an additive.

8. The method of claim 1, wherein the reaction mixture in step (c) is incubated at a temperature of from about 50° C. to about 150° C.

9. The method of claim 1, wherein the reaction mixture in step (c) is incubated at a pH from about 0.5 to about 7.0.

10. The method of claim 1, wherein the reaction mixture in step (c) is incubated for less than 8 hours.

11. The method of claim 1, wherein the reaction mixture in step (c) is incubated for a duration of time ranging from about 12 hours to about 7 days.

12. The method of claim 1, wherein the method results in at least 5% digestion of the total amount of the biopolymer in the sample.

13. The method of claim 1, further comprising adjusting the pH of the reaction mixture to a pH value from about 4.5 to about 7.0, after incubating the reaction mixture.

14. The method of claim 13, further comprising adjusting the temperature of the reaction mixture to a temperature from about 4° C. to about 37° C., after adjusting the pH of the reaction mixture to a pH value from about 4.5 to about 7.0.

15. The method of claim 1, further comprising treating the reaction mixture to remove one of more contaminants, and wherein treating the reaction mixture comprises removing one or more

contaminants from the reaction mixture by chromatography.
16. The method of claim 1, further comprising drying the reaction mixture.
17. The method of claim 1, further comprising storing the prepared sample for a duration of time from about 30 days to about 10 years.
18. The method of claim 4, wherein the composition of step (b) further comprises an additive, wherein the additive is selected from the group consisting of: iodoacetamide (IAA), dithiothreitol (DTT), and any combination thereof.
19. The method of claim 1, wherein the composition of step (b) further comprises an oxidizer.