Automated Determination of Protease for Laundry Compounds

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ABSTRACT

For the determination of the activity of protease for laundry washing compounds preference is given to casein as substrate and measurement of the supernatant at 275 nm after precipitation with trichloroacetic acid. Since sampling rate and precision with the AutoAnalyzer are in this case very low, an automated system based on the principle of the mechanized test tube has been developed. The sampling rate is one sample per minute; the coefficient of variation of a single sample is 2.3% without relation to a standard solution and 1.6% if results are related to a standard solution run simultaneously. If the activity of a sample is determined in threefold, the coefficients of variation are 1.6% resp. 1.1%.

INTRODUCTION

Enzymes, especially proteases, are more and more used in laundry washing compounds. Enzyme preparations are not pure substances and their potency must be expressed in units. A unit of proteolytic activity is usually defined as the degree of digestion of a standard protein substrate in 1 hr by a specific quantity of enzyme preparation under specified conditions of temperature, pH and substrate concentration. However, comparison of two proteolytic enzyme products from different sources, on the basis of the units they are supposed to contain, is uncertain and often misleading. There exists no method of expressing the potency of an enzyme in a universal type of unit meaningful to every use of that enzyme.

The activity of enzymes is dependent on factors such as pH, temperature and substrate concentration. Moreover proteolytic enzymes frequently exhibit a certain substrate specificity and may differ in their action as regards the degree to which proteins are broken down. It is therefore obvious that the potency of enzymes should not be determined under optimal conditions but under those which correlate as closely as possible with the practical conditions under which the enzyme is to be used. For this reason one and the same proteolytic enzyme product will have different units according to its intended use, e.g., for the production of amino acids from proteins, for the purpose of bating hides, as a meat tenderizer, or as a component of washing compounds. There is no predictable correlation between the different units of the same enzyme products, nor between the units for one enzyme and those of other proteolytic enzymes.

The choice of conditions used in determining the activities of proteolytic enzymes destined for washing compounds according to the Delft-method takes the following considerations into account. As a substrate for use in the test we selected casein since this protein, which is present in milk and dairy products, is frequently associated with stains. Another possible substrate could be hemoglobin, but we prefer casein because it can be obtained commercially in a more standardized quality than hemoglobin.

Methods with casein as a substrate are described by Anson (1) and Kunitz (2). We chose Hagihara's modification (3,4) because this method gives a linear calibration curve over a longer concentration range. The enzyme is allowed to act upon a solution of casein for some time under standard conditions simulating washing conditions. The reaction is stopped by the addition of a trichloroacetic acid reagent which precipitates the remaining intact casein (together with some highmolecular breakdown products). After the removal of the precipitate the change in optical density of the solution is determined at 275 nm relative to a blank. The optical density due to the presence of peptide fractions containing animo acids with an aromatic nucleus is a measure of the enzyme activity.

The Delft unit (DU) an arbitrary proteolytic activity unit is defined as follows. An enzyme preparation has a protease activity of 1000 DU/g if 1 ml of a 2% solution of that preparation gives a change in optical density in relation to the blank of 0.400 under the test conditions. The test is conducted in TRIS-buffer (trishydroxymethylaminomethane) at pH 8.5 at 40 C. The incubation time of 40 min allows the determination of the relatively low enzyme content of washing compounds. Alkaline builders, especially sodium tripolyphosphate (STP), can adversely influence the activity of some enzymes. The Delft method measures activity in the presence of STP.

Laundering is normally done in tap water; therefore, a standard water hardness is used in the assay (synthetic tap water, STW, of 15° German hardness). The Delft method for determining protease activity in enzyme preparations and biological washing compounds is described in detail in the Maxatase brochure which is available on request. (Royal Netherlands Fermentation Industries, P.O. Box 1, Delft, Holland.)

AUTOMATION: CONTINUOUS FLOW SYSTEM OR DISCRETE SYSTEM

Several authors have recently published the automation of the method of Kunitz (2) and Hagihara (3,4) with the aid of an AutoAnalyzer. Falck (5) reports an accuracy of ± 2% for 10 samples per hour. According to Hazen et al. (6) the 95% confidence limit is ± 3.5% at 20 samples per hour.

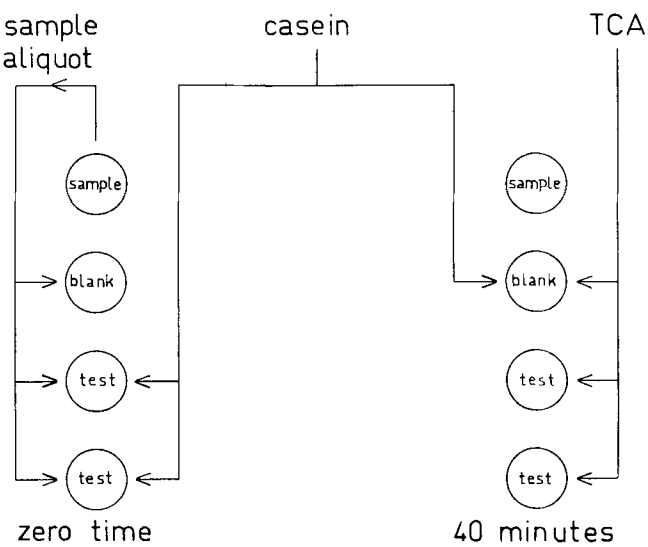

FIG. 1. Pipetting scheme.

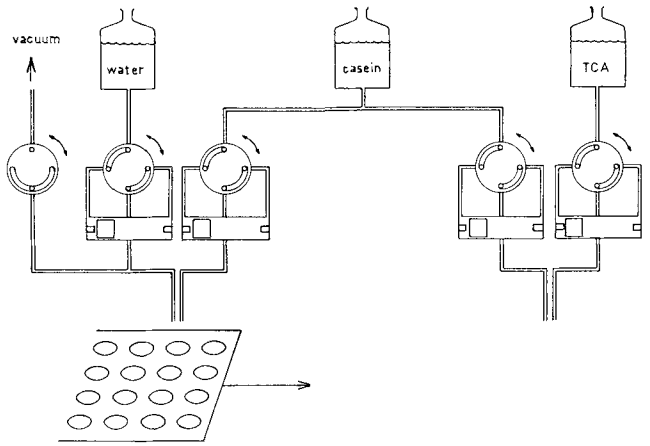

FIG. 2. The tubes are carried past two pipetting stations. At left, ca. 7 ml specimen is sucked into the delivery tube of the sampling pipette. The delivery tube then contains at least 3 ml of uncontaminated sample; 1 ml aliquots are successively dispensed in the 3 neighboring tubes by means of the water-operated lefthand 1 ml plunger. Immediately after this, casein is added to the tests. At right, casein is added to the blank and TCA to tests and blank.

Reisner et al. (7) states that, in general, within a day the range did not exceed ± 2% at a sampling rate of 20 samples per hour. Apparently the number of samples per hour includes the calibrating solutions; the number of unknowns per hour will be considerably lower. The low sampling rate of the AutoAnalyzer is a consequence of the chemistry chosen.

As the samples are only separated by air bubbles, there is inevitably some carry-over, resulting in a contamination of the front of each sample by traces of the preceding sample. The contamination increases with time. For a short reaction time, the distance each sample-volume occupies in the tubing system can be short, because the contamination of the sample is restricted to a very short distance in the system. For a long reaction time however, a much bigger length of sample is contaminated by the preceding sample; to guarantee that at least a fraction of uncontaminated sample reaches the measuring module, each sample must occupy a much longer distance in the flow system. For this reason a long reaction time requires that each sample occupies a long distance in the flow system. This requires a long sampling time; the consequence is a low sampling rate. In this particular case the carry-over is aggravated by the cheese-like character of the precipitate obtained by the addition of trichloroacetic acid.

As we did not want to change the analytical method for the above mentioned reasons, we chose a mechanical system in which aliquots of samples in individual reaction vessels are moved past pipetting stations where reagents are added; such a system can be named a discrete analysis sytem or an automated test tube system.

Although many firms are developing or marketing a discrete analysis system, such a machine was not available in Western Europe at the time we started this investigation. We therefore decided to develop a system suited to our specific needs. The system consists of three separate modules: an incubation and pipetting machine; a battery of centrifuges; and an automated spectrophotometer.

INCUBATION AND PIPETTING MODULE

At zero time, sample aliquots are transferred from the sample tube to three empty, clean reaction tubes. Casein is dispensed into two of the tubes, as the sample test is done in duplicate to obtain a better precision. The remaining reaction tube constitutes the blank (Fig. 1). After 40 min, casein is added to the sample blank and the reaction stopped by addition of trichloroacetic acid to tests and blank. These operations are mechanized by carrying the tubes in racks stepwise past two pipetting stations as indicated in Figure 2. Though the module will not be described in detail, the following points will be discussed: the principle of the automatic pipettes; the rate of the automatic pipettes and the sampling-rate of the module; the mechanical transport of the tubes; and the thermostatic control of the incubation bath.

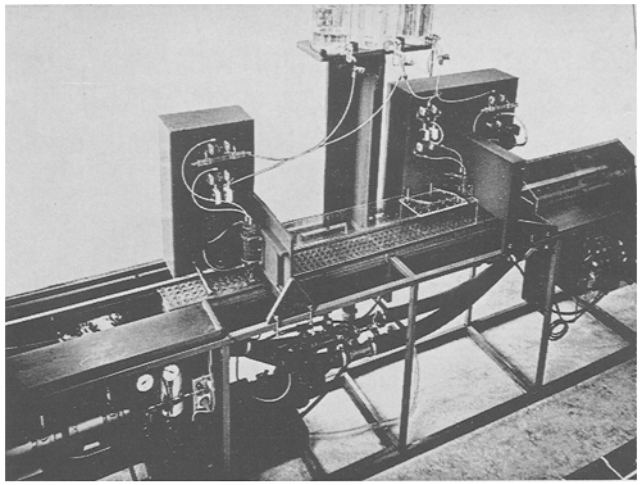

FIG. 3. Incubation and pipetting module.

The Principle of the Automatic Pipettes

The automatic pipettes are based on a patent by I.C.P. Smith (8). Four of these pipettes are shown schematically in Figure 2. The fluid entering from the reservoir pushes a glass piston to the other end of the precision bore barrel, thus dispensing the contained fluid from the delivery jet. On turning the controlling stopcock through 90° by means of an electric motor, the piston travels in the opposite direction and dispenses the same volume of solution. Though this pipette is generally used for dispensing of reagents, it can be used as well for the delivery of sample aliquots, provided the delivery tube is first flushed with sample and then filled with an amount of sample, bigger than the total volume of aliquots that must be dispensed. The pipette at the left in Figure 2 is used to dispense aliquot parts of samples. The delivery jet is first dipped into the sample and flushed by means of the vacuum connection at the extreme left of Figure 2. Enough solution is sucked up to wash away completely the preceding sample. When the vacuum connection is shut off, the deliver jet contains a sufficient amount of uncontaminated sample to dispense the aliquots into blank and test tubes as described.

The Rate of the Automatic Pipettes and the Sampling Rate of the Module

The delivery of an aliquot of a sample (1 ml) and the dispension of casein (5 ml) and TCA (5 ml) take only a fraction of a minute. Because of the necessary additional operations however—lowering, flushing and raising of the sample pipette and successive positioning of the tip of the sample pipette over blank and tests; positioning of the tip of the casein pipette at the first station over two test tubes; and positioning of the tip of the TCA pipette over blank and tests in succession at the second station—nearly one minute is required for each sample. The module therefore moves with a rate of one sample a minute (two tests and one blank).

The Loading and Unloading of the Module and the Mechanical Transport of the Tubes

Use is made of racks which contain 4 rows of 14 tubes. The samples are put in the first row, the second row

FIG. 4. Automated spectrophotometer.

constitutes the blanks; the tubes in the remaining two rows are treated as tests.

The racks enable the operator to load and unload the module with 14 samples at a time and facilitates the mechanical transport of the tubes past the pipetting stations and through the incubation bath (Fig. 3).

Temperature Control of the Bath

Circulation pump, heating element and temperature regulator are situated under the incubation and pipetting module. The water enters at three points evenly divided over one long side of the bath and leaves at two points on the other long side.

THE CENTRIFUGE BATTERY

When a rack has passed the second pipetting station, it is taken from the bath and set aside to aid the coagulation. The reaction tubes are put into one of the centrifuges by hand; one centrifuge has room for 42 tubes, i.e., the tests and blanks of one rack.

The centrifugal acceleration and the duration of centrifugation are critical: the optical density of the supernatant decreases with time. At a centrifugal force of 3500 *g*, the optical density becomes practically constant after 15 min. Differences in centrifugal force, however constitute a major part of the total error of the analysis. This point is further elaborated under Discussion of the Automation.

AUTOMATED SPECTROPHOTOMETER

The automated spectrophotometer is assembled from a LKB "UltroRac," a Beckman DB spectrophotometer and a digital voltmeter and printer, both from Hewlett Packard. After centrifuging, the reaction tubes alias centrifuge tubes are put by hand into the LKB "UltroRac" which functions as sampler for the spectrophotometer. Every 10 sec all tubes move on one step. The sampling probe dips into the solution; the flow cuvette is rinsed by and filled with the solution by means of vacuum and the transmission is printed.

Discussion of the Automation

The saving of labor is considerable. Though the transfer from incubation and pipetting machine to centrifuge and from centrifuge to automated spectrophotometer in done manually, the required manual labor is minimal because of the use of the racks.

The error of the analysis consists of a day-to-day effect (σ day), a rack-to-rack effect (σ rack) and an error for a single sample test within a rack (σ single test). In formula:

$$\sigma \text{ analysis} = \sqrt{\frac{\sigma^2 \text{ day}}{K} + \frac{\sigma^2 \text{ rack}}{nK} + \frac{\sigma^2 \text{ single test}}{nKm}}$$

where K = number of days, n = number of racks per sample in a day, m = number of sample tests within a rack.

By statistical analysis, σ day = 1.1%; σ rack = 1.8% and σ single test = 1.2%.

If a sample is tested as indicated in Figure 1, one sample blank and two sample tests, substitution of m = 2, K = 1 and n = 1 and of the given values of σ day, σ rack and σ single test gives a coefficient of variation of 2.3%.

A better precision is attained if each rack carries a standard solution for calibrating purposes; the standard solution is a solution of a sample with an activity calculated on the basis of determinations over a period of half a year. With this procedure, it is found that σ day = 0.8%, σ rack = 1.1% and σ single test = 1.2%. For m = 2, K = 1 and n = 1, the coefficient of variation is reduced to 1.6%.

The improvement in σ rack is a definite contribution to the improvement of the precision of the analyses. The main reason for this is undoubtedly that, as all tubes from one rack are treated in one centrifuge, differences in centrifugal force exert the same influence on the optical density of samples and standard in one rack.

If a still higher precision is required, the activity is determined by dividing three sample solutions over three racks. Determining the activity of a sample in this way lowers the coefficient of variation to 1.6% without relation to a standard and to as low as 1.1% with relation to a standard.

Infection of casein may lead to high blanks and erratic results. This is avoided by daily flushing of the casein-dispensing-system successively with water, dilute sodium hydroxide and water.

ACKNOWLEDGMENTS

I.J. Stolk constructed the apparatus and C. Liefhebber made the statistical evaluation of the measurements.

REFERENCES

1. Anson, M.L., J. Gen. Physiol. 22:79 (1938).
2. Kunitz, M., J. Gen. Physiol. 30:291 (1947).
3. Hagihara, B., Ann. Rep. Sci. Works Osaka Univ. 2:35 (1954).
4. Hagihara, B., H. Matsubara, M. Nakai and K. Okumuki, J. Biochem. (Japan) 45:185 (1958).
5. Falck, A., "Automation in der Analytischen Chemie," Internationales Technicon Symposium, Frankfurt, M, 1964 p. 263.
6. Hazen, G.G., Y.A. Hause and J.A. Hubicki, Ann. N.Y. Acad. Sci. 130:761 (1965).
7. Reisner, W., and H. Uhlig, Z. Am. Chem. 215:190 (1966).
8. Smith, I.C.P., British Patent 613, 334 (1946).

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