The Raw Material, Finished Products and Dust Pad Analysis of Detergent Proteases Using a Small Synthetic Substrate

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A new method is presented that utilizes p-nitroanilide substrates to quantify proteolytic activity of detergent enzymes. The method is contrasted to the dimethylcasein method currently in use by detergent industries, and the advantages of the new method and its potential for monitoring enzyme dust in factories are discussed.

Currently, the detergent industry incorporates proteolytic enzymes into many of their detergent products. To ensure these products can be safely manufactured (1-3), guidelines designating maximum airborne enzyme dust limits in factories have been established by the American Conference of Governmental Hygienists (ACGIH) (4). These guidelines have been met through use of a method developed specifically to monitor airborne protease dust (5-6). The method relies on an activity analysis produced from protease digestion of N,N-dimethylcasein (DMC) and the resultant reaction of new free amino groups of the enzyme-released peptides with trinitrobenzenesulphonic acid (TNBSA) to produce colorimetrically detectable Meisenheimer complexes (7).

While this method is adequate for levels of enzymes for product analyses or manufacture of pure enzyme receipt, our experience and historical data concerning manufacture of enzyme-containing detergent products indicate that maximum dust limits for factories can be controlled at levels six to 10 times lower than those recommended by the ACGIH. The enzyme dust limit for our manufacturing facilities has, therefore, been set to a level that is 10 times lower than the level recommended by the ACGIH. With this reduction in dust limits, the DMC/TNBSA methodology is barely sensitive enough for our needs. We have observed that approximately one of every two samples collected has enzyme levels below the method's detection limits. Therefore, a new program to increase analytical sensitivity as well as to streamline and simplify our existing methodology has been instituted.

Efforts from this program have produced a new method which currently is being used in our factories. This method is an extremely sensitive activity assay that uses p-nitoranilide (pNA) substrates. Use of one of these, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylanyl p-nitroanilide, was first reported as a sensitive reagent to detect chymotrypsin activity (8). We have observed that this substrate, as well as some of the other p-nitroanilides, is quite applicable to detection of detergent proteases in the presence of high levels of surfactants. Within this paper, we will present this new pNA method, contrast it to the DMC/TNBSA assay and illustrate its application for monitoring protease dust in detergent factories.

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MATERIALS AND METHODS

Materials. Dimethylsulfoxide, trishydroxymethyl aminomethane, calcium chloride dihydrate, sodium thiosulfate pentahydrate, phenylmethylsulfonyl fluoride, N-succinyl-L-alanyl-L-alanyl-L-alanyl p-nitroanilide, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl p-nitroanilide, and N-succinyl-L-alanyl-L-alanyl-L-valyl-L-alanyl p-nitroanilide were purchased from Sigma Chemical Company (St. Louis, Missouri). Sodium borate decahydrate, sodium phosphate (monobasic), and anhydrous sodium sulfite were obtained from Fisher Scientific Co. (Cincinnati, Ohio). N,N-dimethylcasein was obtained through Gallard Schlesinger Chemical Co. (Carleplace, New York). All enzymes tested were detergent proteases obtained directly from industrial commercial enzyme suppliers.

pNA method. Except where changes are noted, all pNA assays were conducted by using a 0.1 mol/l Tris, 0.01 mol/l $CaCl_2 \bullet 2H_2O$, 0.02 mol/l Na_2SO_3, pH 8.3 stock buffer solution, a 0.2 mol/l pNa substrate (in DMSO) stock solution, and appropriate enzyme dilutions in the stock buffer solution. Reactions were initiated by addition of 50-150 μl of the stock substrate into an assay volume of five ml with thorough mixing. Throughout the reaction period, the solution temperature was maintained at 37 C. Tight temperature control is a necessity as reaction rates are influenced by changes in temperature. Reactions were stopped after approximately six min by addition of 50 μl of a 1% solution of phenylmethylsulfonyl fluoride in methanol (PMSF). Depending on solution turbidity (e.g., presence of insoluble dust and/or portions of dust pad), this solution may be filtered through silanized glass wool. The solution absorbance is measured spectrophotometrically at 410 nm. Enzyme activity is quantified by relation of sample absorbance to response curves of absorbance vs enzyme concentration for standards of each enzyme group.

Dust analysis and DMC method. DMC analysis was conducted for enzyme activity using the DMC method described by Dunn and Brotherton (5). Dust collections were accomplished using glass fiber filters (Whatman GF/C, Fisher Scientific Co., Cincinnati, Ohio) and following the method described.

Enzyme activity. All protease activities are expressed as Anson Unit activity where one Anson Unit is equivalent to the amount of protease digested hemoglobin/min that is not precipitated by trichloroacetic acid and gives the same color by phenol reagent as does one milliequivalent of the amino acid, tyrosine (9).

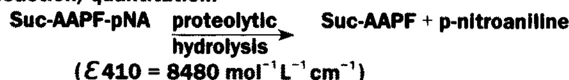
RESULTS AND DISCUSSION

Figure 1 gives a brief overview of the two analytical methodologies, DMC and pNA. The most commonly used p-nitroanilide (pNA) substrate is the synthetic

pNA Method

Substrate:
Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide

Reaction/quantitation:



DMC Method

Substrate: **Dimethylcasein**

Reaction/quantitation:

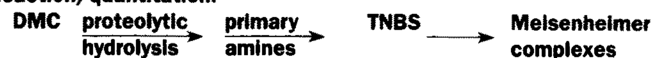


FIG. 1. Overview of pNA and DMC/TNBS assays.

TABLE 1

pNA Assay Conditions

Buffer:	0.1 mol/l Tris 0.01 mol/l $CaCl_2$ 0.02 mol/l $Na_2S_2O_3$
pH:	8.3
Substrate concentration:	2.0×10^{-3} mol/l (50 μl of a 12.5% w/v solution of pNA in DMSO into a 5-ml assay volume)
Time:	6 min
Enzyme concentrations:	required concentration to produce a 0.6-OD response at 410 nm Group A 5.0×10^{-7} AU[a]/ml Group B 1.6×10^{-6} AU/ml Group C 4.4×10^{-6} AU/ml Group D 2.7×10^{-6} AU/ml

[a]AU, Anson Unit, activity as defined in Materials and Methods. Typical assay volume of 5 ml implies a total of 2.5×10^{-6} AU of protease necessary for quantitation or approximately 160 μg of a typical protease-containing detergent product. Product concentration in assay is 30 ppm.

tetrapeptide, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl p-nitroanilide (Suc-AAPF-pNA). Hydrolytic action on this substrate cleaves the phenylalanyl p-nitroanilide amide bond yielding the yellow chromophore p-nitroaniline. Quantitation of this activity follows from the measurement of absorbance at 410 nm. In the N,N-dimethylcasein (DMC) assay, the substrate is the protein, dimethylcasein (DMC).

Proteolytic action of this substrate results in the generation of primary amines. Therefore, quantitation of the proteolytic activity follows from the determination of primary amines. For this assay, reaction of the primary amines with trinitrobenzene sulfonate (TNBS) forms Meisenheimer complexes - yellow chromophores that can be detected and quantitated at 420 nm.

An important difference between these two assays is the quantitation step. In the pNA assay, enzyme quantitation results directly from the proteolytic action. In the DMC assay, quantitation is indirect because it requires a chemical reaction separate from the proteolytic hydrolysis. This indirect quantitation is more susceptible to interferences. For example, primary amine interference is a serious shortcoming of the DMC assay.

Table 1 shows the typical assay conditions used in the pNA assay. The assay is buffered at pH 8.3 with 0.1 mol/l Tris buffer containing 0.01 mol/l each of calcium chloride and 0.02 mol/l of sodium thiosulfate. The substrate (Suc-AAPF-pNA) concentration is 2×10^{-3} mol/l. This is a sufficient substrate concentration to maintain a linear response with time for all the proteases tested (i.e., all enzymes are assayed at close to substrate saturating conditions). This is also a sufficient substrate concentration if completely hydrolyzed to generate an optical density of 17 at 410 nm. In a typical assay time of six min, the O.D. (410 nm) is kept below 0.6, which means that less than 5% of the available substrate is consumed.

Enzymes used in this study belong to four distinct groups, each of which is represented in Table 1 by A, B, C or D. Currently, all commercially available detergent proteases belong to one of these four groups. For example, the protease Subtilisin Carlsberg (Subtilopeptidase A, EC 3.4.4.16) would belong to Group A. The approximate enzyme concentrations needed to generate a response of 0.6 O.D. (410 nm) are shown for each of the four groups. Noteworthy is that the enzyme concentrations are different. This illustrates the kinetic differences between the enzyme groups mentioned above.

To put these enzyme concentrations into perspective, consider a hypothetical product containing 1% of a material that is 1.5 Anson Units/g (AU/g) of Group A protease. The 0.6 O.D. (410 nm) response in a total assay volume of five ml would require 160 μg of product. This means the typical product concentrations in the assay are less than 30 ppm. The assay sensitivity allows one to work, therefore, at very dilute product concentrations - dilute enough that virtually all matrix effects are diluted out. Typically, a single calibration curve can be used to quantitate proteolytic activity across all sample matrices.

Table 2 compares some of the more important aspects of the pNA and DMC assays. These comparisons are for the Group A proteases. Note, the pNA assay is over three times faster than the DMC assay at an enzyme concentration 1/10 that of the DMC assay. This is a real 30-fold increase in sensitivity and translates into significantly higher productivity when using the pNA assay. This higher sensitivity, coupled with the very low blank (analytical response without protease) and lack of known interferences, makes the pNA assay a significant improvement over the DMC assay.

The DMC assay suffers serious interference from nonenzymatically generated primary amines. This interference, combined with typically high blank values,

TABLE 2

pNA and DMC Assay

	pNA	DMC
Time	6 min	25 min
Enzyme concentration	1-5 × 10^{-7} AU[a]/ml	1-5 × 10^{-6}AU/ml
Typical blank	< 0.01 $OD410_{nm}$	> 0.2 $OD420_{nm}$
Interferences	None	Primary amines
Accuracy	Excellent	Excellent (in the absence of 1° amines)
Precision	< 0.5% RSD liquid and granular products	5% RSD over all products
Advantages	Quick Accurate and precise Stable reagents Sensitivity (little/no product matrix effects) Response linear with time pH independent	Little enzyme-to-enzyme variation
Disadvantages	Significant enzyme-to-enzyme variation	Time consuming High blank Unstable reagents Primary amine interference

[a]AU, Anson Unit, activity as defined in Materials and Methods.

severely limits this assay when it is used to quantitate low levels of proteolytic activity. This limitation is most serious when using the DMC assay to measure airborne enzyme levels.

The accuracy and precision of the pNA assay are excellent. Overall, the pNA assay is far superior to the DMC assay in this regard, primarily because of the DMC assay's primary amine interference and high blank values.

Table 3 summarizes the results of assays conducted for the different time periods 6, 60 and 1,440 min respectively. For each time, a different range of Group A enzyme standard concentrations was used and a four-point calibration line was constructed. Activity is expressed as a function of the O.D. (410 nm) generated/unit time. The slopes of these calibration lines are a direct measure of the analytical response and should be independent of enzyme concentration and of time.

Note for each calibration line the analytical response is linear, as can be deduced from the linear correlation coefficients. Also note between each calibration line, the slopes (a measure of the analytical responses) are very similar - identical within 12%. The response, therefore, is linear with time, which makes the analytical assay much more flexible in dealing with enzyme concentration and assay time. This is a great advantage when working with samples of completely unknown activity (such as enzyme dust pads). A calibration curve can be constructed (6-min assay), the unknown sample allowed to react until there is suitable color development and the enzyme activity of the unknown can be determined by factoring time into the response.

Table 4 describes the pH dependence of the different enzyme groups in the assay. Here, the response at pH values 9.3 and 9.7 are normalized to the observed re-

TABLE 3

pNA Assay Linearity Over Several Concentration Ranges of Enzyme

Reaction time (min)	Group A enzyme concentration (AU/ml)	Slope[a] AU/ml / O.D./min	Correlation coefficient
6	2-10 × 10^{-7}	6.61 × 10^{-6}	0.991
60	2-10 × 10^{-8}	6.85 × 10^{-6}	0.996
1,440	.75-2.5 × 10^{-9}	7.32 × 10^{-6}	0.994

[a]Slope, concentration of enzyme in AU/ml that will produce a rate of 1.0 O.D./min.

TABLE 4

pNA Assay and pH Dependence

	Relative response for assay pH[a]		
Enzyme group	8.3	9.3	9.7
A	1.00	1.05	0.95
B	1.00	0.88	0.90
C	1.00	1.09	1.01

[a]Relative assay responses are normalized to response at pH 8.3.

TABLE 5

Comparison of Relative pNA and DMC Assay Sensitivity Limits for Dust Pads

Method	per assay[a] μg product @0.015 AU[d]/g	per assay[a] μg CAPE[c] @65 AU/g	per dust pad[b] μg product @0.015 AU/g	per dust pad[b] μg CAPE[c] @65 AU/g
pNA method				
6 min	160	0.037	800	0.185
60 min	16	0.0037	80	0.0185
1,440 min	0.67	0.00015	3.33	0.00075
DMC method				
25 min	320	0.074	1,600	0.370

[a]Assumes an assay volume of 5 ml.
[b]Assumes an assay volume of 5 ml with a total dust pad elution volume of 25 ml.
[c]CAPE, Crystalline Active Pure Enzyme. pNA sensitivity limit for airborne CAPE is 10×10^{-12} g/m^3 @ 75 m^3 sampling volume.
[d]AU, Anson Unit, activity as defined in Materials and Methods.

sponse at pH 8.3 for enzyme groups A-C. As can be seen, again within 12%, the analytical response is independent of the assay pH. This is an advantage in that small fluctuations in assay pH do not affect the accuracy of the assay.

Interestingly, all these enzymes are reported to have a substantial pH dependence of activities on other substrates (hemoglobin, dimethylcasein, Azocol, etc.). Yet this is not observed with the pNA substrate. As the pNA substrate does not change charge in the alkaline pH region, these pH activity differences on other substrates may well be caused at least partially by pH-induced charge changes on the substrates.

Clearly, the pNA methodology is a very sensitive assay well-suited for the analyses of small quantities of proteolytic activity. Sensitivity is the primary prerequisite for the quantitation of enzyme in factory dust collected on dust pads. Table 5 shows the sensitivity limits for this methodology applied to the quantitation of proteolytic activity on dust pads. The relative sensitivities of the pNA and DMC methodologies are compared on a per assay basis as well as on a per dust pad basis. For illustration purposes, the pNA sensitivities of the three reaction times in this comparison are based on calculations made using the six-min reaction data, and the sensitivity limit shown for the DMC assay is based on the 25-min assay described in Materials and Methods. The pNA sensitivities shown in this comparison are the quantities of proteolytic activity (Group A) that generate a response of 0.6 O.D. (410 nm) in either a 6-60-, or 1,440-min (24-hr) time period and therefore are very conservative estimates of the actual sensitivity limits of the assay. Even with this conservative estimate, however, the assay is capable of quantitating the proteolytic activity in 670 ng of product or of 150 pg of Crystalline Active Pure Enzyme (CAPE) if one allows for a 24-hr incubation period. This assumes a product specific activity of 0.015 Anson Units/g (AU/g) and a pure enzyme specific activity of 65 AU/g of CAPE (one AU is defined in Materials and Methods). If a high volume air sampler is used, these sensitivities translate to the quantitation of 10 pg of CAPE/m^3 of sampled air. This is far better sensitivity than any other available methodology and allows quantitation of airborne subtilisins at levels approximately 600-fold lower than the Threshold Limit Value (TLV) set by the American Conference of Governmental Industrial Hygienists (4).

There is also a potential disadvantage of the pNA versus the DMC assay. This is the enzyme-to-enzyme variation. As mentioned earlier, the different enzyme groups do respond differently in the assay. This can be a disadvantage when dealing with an unknown enzyme or enzyme mixtures. The response variances of the four enzyme groups are indicative of different kinetic behavior and can be characterized through comparisons of the relative rates vs substrate concentration for the four enzyme groups. If one assumes a Michaelis-Menton kinetics model, these comparisons can be used to derive kinetic constants which quantitatively describe these kinetic differences. Table 6 shows the derived kinetic constants for the four enzyme groups with the substrate, Suc-AAPF-pNA. The constants are K_m, the Michaelis constant, and k_{cat}, the enzyme turnover number. K_m is related empirically to the binding affinity of the enzyme for the substrate. Rigorously defined, it is the substrate concentration required to achieve 1/2 maximum velocity of the enzymatic reaction. Therefore, the larger the value of K_m, the less affinity the enzyme has for the substrate. k_{cat} Is the number of substrate molecules converted into product/unit time. When the enzyme is fully saturated with substrate, k_{cat} is a direct measure of the analytical response. This means that enzymes in Group A have 740/230 or 3.2 times higher response with this substrate than do the enzymes in Group B.

TABLE 6

Derived Kinetic Constants of Each Enzyme Group for Suc-AAPF-pNA Substrate

Enzyme group	K_m (mol/l)	k_{cat} (s^{-1})
A	2.5×10^{-4}	740
B	9.0×10^{-4}	230
C	9.1×10^{-4}	84
D	1.4×10^{-4}	137

TABLE 7

Relative Protease Responses to Different pNA Substrates[a]

Protease	Substrate	Relative time to produce equal response
A	Suc-AAA-pNA	313
	-AAVA-	83.3
	-AAPF-	1.0
B	Suc-AAA-pNA	37.0
	-AAVA-	23.4
	-AAPF-	3.2
C	Suc-AAA-pNA	97.8
	-AAVA-	60.4
	-AAPF-	8.8
D	Suc-AAA-pNA	1,482
	-AAVA-	115
	-AAPF-	5.4

[a]Response based on equal moles of each protease.

This also means that one must use like enzymes (from the same group) for method calibration and quantitation.

This difference in response can be used, however, as an important diagnostic tool. The different responses for the four enzyme groups across three different synthetic substrates are given in Table 7. The substrates are N-succinyl-L-alanyl-L-alanyl-L-alanyl p-nitroanilide (Suc-AAA-pNA) and N-succinyl-L-alanyl-L-ananyl-L-valyl-L-alanyl p-nitroanalide (Suc-AAVA-pNA) in addition to the now familiar Suc-AAPF-pNA. Here is shown the reaction time necessary to produce an equal response (O.D. 410 nm)/mole of enzyme for each of the enzyme groups. The responses all are normalized to the Group A response on Suc-AAPF-pNA. Note the relative responses of Groups B, C and D to the Suc-AAPF-pNA substrate reflect the differences in k_{cat} values in Table 6. Note also the responses are different for the different enzyme groups across the other substrates as well.

Therefore, by assaying an unknown protease sample using these three different substrates, one can identify the group the unknown protease belongs to and quantitate the proteolytic activity present. One can also use a similar treatment to quantitate the respective components in enzyme mixtures from different enzyme groups. This potential disadvantage of different responses can, therefore, be used as a powerful diagnostic tool.

The pNA method presented in this work offers a number of significant advantages over current DMC/TNBSA methodology. The sensitivity is high enough to "dilute" out virtually all product matrix effects. The shelf life of the pNA assay buffer and substrate in dimethyl sulfoxide is greater than one mo. Therefore, unlike the DMC assay, reagents can be prepared far in advance and used on a moment's notice as needed. The pNA method offers good accuracy and precision. This is especially true when measuring low levels of proteolytic activity and/or in the presence of primary amines. It is also a relatively fast procedure. On a per assay basis, the pNA assay is at least three times faster than the DMC/TNBSA, and if one takes into account reagent preparation and assay set-up, it is more than 10 times faster. The method is not affected by primary amines or other product matrix components that normally offer interference in the DMC analysis. Activity responses are extremely linear over three orders of magnitude and pH independent over a range of 8 to 10. The simplicity of this method also facilitates automating the procedure. There are a number of advantages to the new pNA methodology over the existing DMC method. These advantages include, high sensitivity; good accuracy and precision; stable reagents; no product matrix effects; linear response with time; pH independent response; relatively fast and simple, and significant enzyme-to-enzyme variation as a diagnostic tool. The last point, although a potential disadvantage, can be viewed as an advantage as well, especially when identifying enzyme types or quantifying mixed enzyme systems.

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