Evaluation of the Progress of Protein Hydrolysis

This unit describes several techniques that can be used to evaluate the progress of protein hydrolysis by using the concept of degree of hydrolysis (DH). The DH is defined as the percentage of hydrolyzed peptide bonds. The techniques described here are based on the assumption that a free amino group and a free carboxyl group are released every time a peptide bond is hydrolyzed. They quantify the increase in the concentration of such groups as a variable to evaluate the progress of hydrolysis.

The ninhydrin reaction (see Basic Protocol 1), the TNBS reaction (see Alternate Protocol 1), the fluorescamine reaction (see Alternate Protocol 2), and formol titration (see Alternate Protocol 3) all evaluate released amino groups by comparing the amounts of free amino groups before and after hydrolysis. The first three methods are spectro-photometric techniques, whereas the fourth is a potentiometric technique. The first and second are chromogenic techniques, whereas the third is fluorometric. These techniques are usually performed as time-course experiments. As the hydrolysis reaction proceeds, aliquots (samples) of the reaction are taken periodically and treated with a test reagent. Products of this reaction are proportional to the amount of free amino groups at each time point.

One additional technique presented is the pH-stat technique (see Basic Protocol 2). This is a kinetic technique used to evaluate the progress of hydrolysis. The hydrolysis reaction is followed in the same reaction vessel at theoretically infinitesimal increments. This gives a graph of the progress of the hydrolysis. The pH-stat technique is useful for evaluating the progress of an enzyme-catalyzed protein hydrolysis process on an industrial scale, where the degree of hydrolysis is the most important variable in ensuring that products with desirable functional and organoleptic properties are obtained.

STRATEGIC PLANNING

Protein hydrolysates are usually produced by limited enzymatic hydrolysis of protein molecules in foodstuff, yielding polypeptides that are smaller in molecular mass. Protein hydrolysis has several aims. The most common is to make the protein moiety of a foodstuff soluble by reducing the size of the peptides. Solubilization simplifies isolation of the protein moiety by physical means. Protein hydrolysis has also been applied to improve the functional, organoleptic, and nutritional value of a foodstuff. Advances in the technology of protein hydrolysate production has allowed the use of unconventional protein sources for animal and human food.

The following test parameters must be defined for the production of a protein hydrolysate: the mass of the hydrolysis mixture (M in g or kg), the substrate (S in % w/w) where protein concentration ($N \times f_N$) is determined by the Kjeldahl method (*UNIT B1.2*), the enzyme/substrate ratio (E/S, as activity units per gram or kilogram of substrate), the pH, and the temperature (°C). Also, the conditions needed to terminate hydrolysis must be defined. Because hydrolysis is typically allowed to proceed until a given degree of hydrolysis (DH) value is reached, the reaction is terminated by a sudden inactivation of the enzyme. Sometimes, termination is achieved by changing the pH. If a reaction is performed under alkaline conditions, lowering the pH to 4.0 to 4.5 concludes hydrolysis. For thermosensitive enzymes, rapidly heating the reaction mixture is suitable. In both cases, preliminary assays should be performed to determine satisfactory conditions, especially to determine the appropriate time at which to inactivate the enzyme under real reaction conditions.

Biochemical Compositional Analyses of Proteins

When producing protein hydrolysates, the next step is often to separate the solubilized protein from the nonhydrolyzed material. Centrifugation or filtration yields soluble protein and a precipitate or sludge.

NINHYDRIN REACTION TO DETERMINE DEGREE OF PROTEIN HYDROLYSIS

In the following protocol, amino acids are reacted with ninhydrin hydrate at pH 5 and 100°C for a standard period of time, yielding a purple-blue compound, the ammonium salt of diketohydrindylidene-diketohydrindamine, as the major product. The structure of the reagents and the reaction is shown in Figure B2.2.1. Amino acids generally react with ninhydrin to yield carbon dioxide, ammonia, and, usually, an aldehyde of one carbon atom fewer than the original amino acid. Ninhydrin also reacts with ammonia and primary amines. The absorbance of the purple-blue product is measured at 570 nm. Proline and hydroxyproline give a yellow product whose absorbance is measured at 440 nm (Fig. B2.2.2).

NOTE: A variety of assay conditions can be used here; this protocol is specific for Alcalase. Many other enzymes can be used, as well as other percentages of protein and other enzyme/substrate ratios.

Materials

Substrate: sample solution containing native protein
Enzyme (e.g., Alcalase 0.6L or Alcalase 2.4L; Novo Nordisk Biochem), adjusted to desired reaction pH using HCl or NaOH
Water or buffer at desired pH and temperature
3 N HCl
Standard solution: 1.5 mM leucine (1.5 meq NH_2/g) in 50% (v/v) isopropanol
Standard solution: 1.5 mM proline or hydroxyproline (1.5 meq NH_2/g) in 50% (v/v) isopropanol
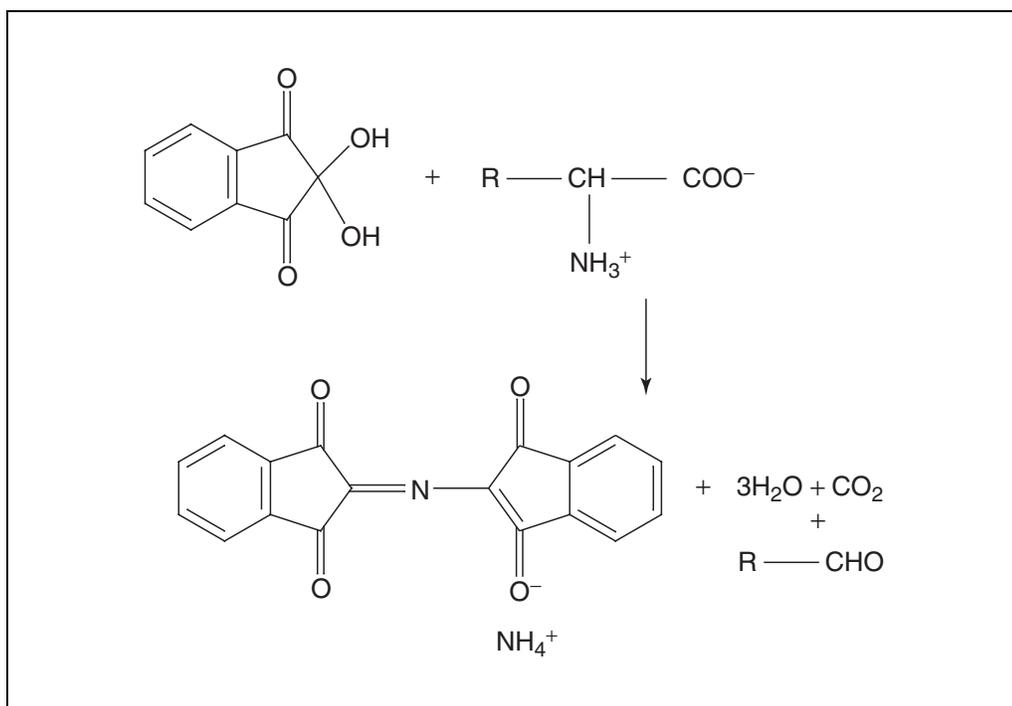Ninhydrin reagent (see recipe)

Figure B2.2.1 Reaction of ninhydrin with an amino acid, producing the purple-blue product.
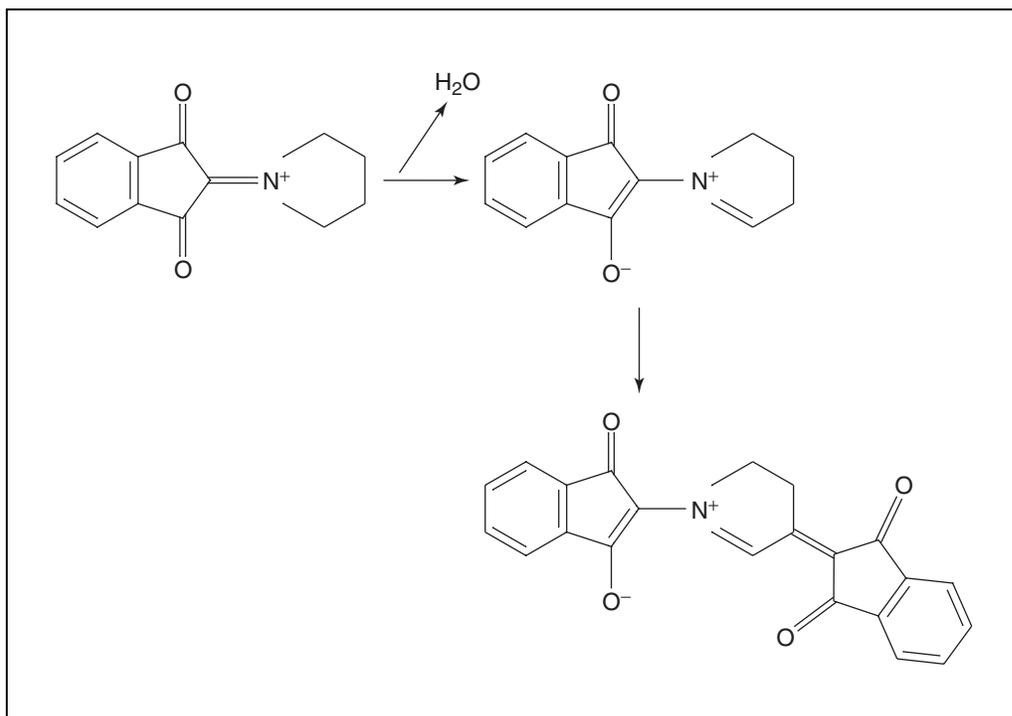
Figure B2.2.2 Reaction of ninhydrin with proline or hydroxyproline, producing the yellow product.

Water baths at 85° (optional) and 100°C
13 × 100–mm glass test tubes
Spectrophotometer (visible range, 440 to 570 nm)
Glass spectrophotometer cuvettes

Prepare hydrolysis samples

1. Determine the appropriate amounts of substrate and enzyme to use for the hydrolysis reaction. Use an amount of substrate that gives protein at 8% (w/w) of the total reaction and calculate enzyme at a ratio of 0.012 Anson U/g protein. For example, for a 100-ml Alcalase hydrolysis reaction, use:

> substrate containing 8 g protein
> 0.16 g Alcalase 0.6L or 0.04 g Alcalase 2.4L
> buffer to 100 ml.

The amount of protein in the substrate solution can be determined by using the Kjeldahl method (UNIT B1.2) to determine the nitrogen concentration (N) and then multiplying this by a factor of 6.25 (f_N).

The mass of enzyme in grams (M_E) is calculated $M_E = M_P \times (A/E)$, where (M_P) is the mass (in g) of protein in the substrate, A is the suggested enzyme activity in Anson units/gram (0.012 AU/g), and E is the actual enzyme activity specified by the manufacturer. For Alcalase, 0.6L and 2.4L correspond to 0.6 and 2.4 AU/g, respectively. Thus $M_E = 8$ g $\times$ (0.012 AU/g ÷ 0.6 AU/g) = 0.16 g for 0.6L, or 0.04 g for 2.4L.

2. Mix substrate in water or an appropriate buffer at the desired temperature and pH.

Every sample should be analyzed with appropriate replicates.

3. Before adding enzyme, transfer a 1.0-ml sample to a 13 × 100–mm glass test tube to be used for determining free amino groups in the raw material.

4. Add enzyme to the remaining substrate solution and stir mixture throughout the course of the reaction.

5. Take 1.0-ml samples at suitable time intervals during the reaction and transfer to glass test tubes. Immediately inactivate each sample by lowering the pH value to 4.0 with 3 N HCl (10 to 20 µl) or by transferring the sample to a water bath at 85°C for at least 15 min.

   If HCl is used to stop the reaction, it should also be added to the control (step 3) and to the standards and blank (steps 6 and 7) to maintain consistent dilution of amino equivalents.

Perform ninhydrin reaction

6. Prepare a series of 1-ml calibration standards by diluting 1.5 mM leucine standard solution containing 0.01 to 0.15 amino meq/g in the same solvent that was used to prepare the sample protein. Prepare a series of proline or hydroxyproline standards in the same manner.

   As with the samples, the standards and blank should be prepared in appropriate replicates.

7. Prepare a blank containing 1 ml solvent alone.

8. Add 0.2 ml ninhydrin reagent to each tube (steps 3, 5, 6, and 7).

9. Heat 10 min at 100°C and cool to room temperature.

Analyze

10. Measure absorbance at 570 nm for any amino acid, and at 440 nm for proline and hydroxyproline.

11. Create separate calibration curves for leucine and proline by plotting A_{570} or A_{440}, respectively, versus equivalent amino acid standard concentration.

12. For each time point, determine the concentration equivalents of amino groups in the samples by extrapolating from each standard curve.

13. For each time point, separately determine the amount of released amino groups (h) by subtracting the value of the corresponding unhydrolyzed control (step 3) in the corresponding standard curve.

    This gives the number of equivalents of peptide bonds hydrolyzed (h), expressed as meq/g protein from the following equation:

    $$h = (A \times b)/m$$

    where A is the absorbance at 570 or 440 nm and b and m are the y intercept and slope of the calibration curve. To get h, sum the values of h from 570 and 440.

14. Determine the degree of hydrolysis at each time point from the following equation:

    $$DH = h/h_{tot} \times 100\%$$

    where h_{tot} is the total amount of peptide bonds.

    When h_{tot} remains unknown, 8 amino meq/g protein is a good estimate (Adler-Nissen, 1986).

TNBS REACTION TO DETERMINE DEGREE OF HYDROLYSIS

Trinitrobenzenesulfonic acid hydrate (TNBS) reacts with amino acids, yielding a yellow product (Fig. B2.2.3) whose absorbance is measured at 340 nm. TNBS reacts only with amino groups in their unprotonated state. TNBS binds to amino acids in two steps: (1) a fast reaction with low affinity, and (2) a slow reaction with relatively high affinity. For practical purposes, the two-step reaction should be considered as one. TNBS reacts with primary amines under slightly alkaline conditions, and lowering the pH stops the reaction.
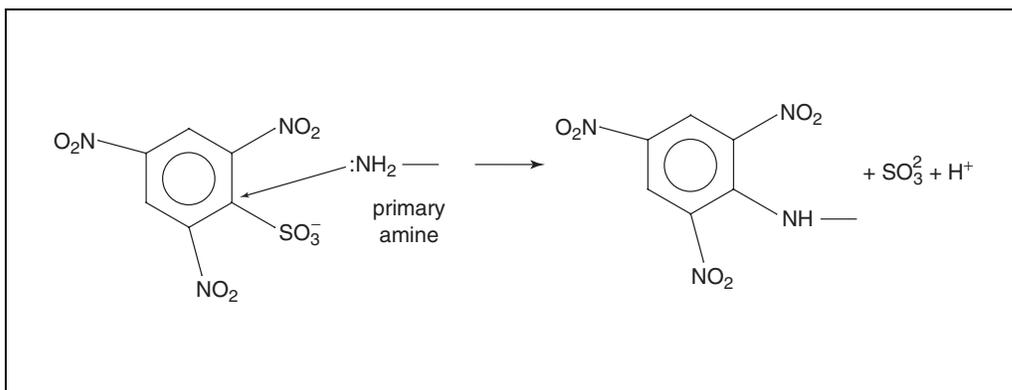
Figure B2.2.3　TNBS reaction.

Additional Materials (also see Basic Protocol 1)

　　1% (v/v) SDS (*APPENDIX 2A*)
　　0.2 M sodium phosphate buffer, pH 8.2 (*APPENDIX 2A*)
　　2.5 mM leucine standard in 1% SDS
　　0.1% (w/v) trinitrobenzenesulfonic acid dihydrate (TNBS) in deionized water
　　　(prepare immediately before use and protect from light)
　　0.1 N HCl

　　15×85–mm and 16×125–mm glass test tubes
　　50° and 75°C shaking water baths
　　Spectrophotometer (visible range; 340 nm)

Prepare hydrolysis samples

1. Determine the appropriate amounts of substrate and enzyme to use for the hydrolysis reaction. Use an amount of substrate that gives protein at 8% (w/w) of the total reaction and calculate enzyme at a ratio of 0.012 Anson U/g protein. For example, for a 100-ml Alcalase hydrolysis reaction, use:

　　　substrate containing 8 g protein
　　　0.16 g Alcalase 0.6L or 0.04 g Alcalase 2.4L
　　　buffer to 100 ml.

   The amount of protein in the substrate solution can be determined by using the Kjeldahl method (UNIT B1.2) to determine the nitrogen concentration (N) and then multiplying this by a factor of 6.25 (f_N).

   The mass of enzyme in frams (M_E) is caluculated $M_E = M_P \times (A/E)$, where (M_P) is the mass (in g) of protein in the substrate, A is the suggested enzyme activity in Anson units/gram (0.012 AU/g), and E is the actual enzyme activity specified by the manufacturer. For Alcalase, 0.6L and 2.4L correspond to 0.6 and 2.4 AU/g, respectively. Thus $M_E = 8$ g $\times$ (0.012 AU/g $\div$ 0.6 AU/g) = 0.16 g for 0.6L, or 0.04 g for 2.4L.

2. Mix substrate and appropriate buffer at the desired temperature and pH.

   Every sample should be analyzed with appropriate replicates.

3. Before adding enzyme, take a 0.25-ml sample and transfer to a 15×85–mm glass test tube containing 2 ml of 1% SDS and incubate 15 min at 75°C.

   This unhydrolyzed control will be used for evaluation of free amino groups in the raw material.

4. Add enzyme to the remaining substrate solution and stir mixture throughout the course of the reaction.

**Biochemical
Compositional
Analyses of
Proteins**

B2.2.5

5. Take 0.25-ml samples at suitable time intervals during the reaction. Immediately transfer each sample to a test tube containing 2 ml of 1% SDS and keep at 75°C in a shaking water bath for 15 min.

   This inactivates the enzyme and disperses the protein hydrolysate.

Perform TNBS reaction

6. Transfer 0.25 ml from each sample (step 5) and control (step 3) to separate 16 × 125–mm glass test tubes containing 2 ml of 0.2 M sodium phosphate buffer.

7. Prepare six to ten calibration standards by diluting 2.5 mM leucine standard containing 0.25 to 2.5 amino meq/g in sodium phosphate buffer at a total volume of 2.25 ml.

   As with the samples, the standards and blank should be prepared in appropriate replicates.

8. Prepare a blank containing 0.25 ml of 1% SDS in 2 ml sodium phosphate buffer.

9. Add 2 ml of 0.1% TNBS solution to each tube, vortex, and incubate in the dark at 50°C for 60 min.

10. Stop the reaction by adding 4.0 ml of 0.1 N HCl. Allow samples to reach room temperature and read absorbance at 340 nm.

Analyze

11. Create a calibration curve by plotting A_{340} versus concentration of the standards.

12. Determine the degree of hydrolysis (see Basic Protocol 1, steps 12 to 14).

ALTERNATE PROTOCOL 2

FLUORESCAMINE REACTION TO DETERMINE DEGREE OF HYDROLYSIS

Fluorescamine reacts with primary amines to form fluorophores (see Fig. B2.2.4) that are excited at 390 nm and fluoresce at 475 nm. Peptides react with fluorescamine at pH 7.0, giving higher fluorescence than amino acids, which have maximum fluorescence at pH 9. The reaction proceeds rapidly with primary amines at 25°C. The resulting fluorescence is proportional to the amine concentration. The fluorophores are stable for several hours. A negligible interference is produced with ammonia.
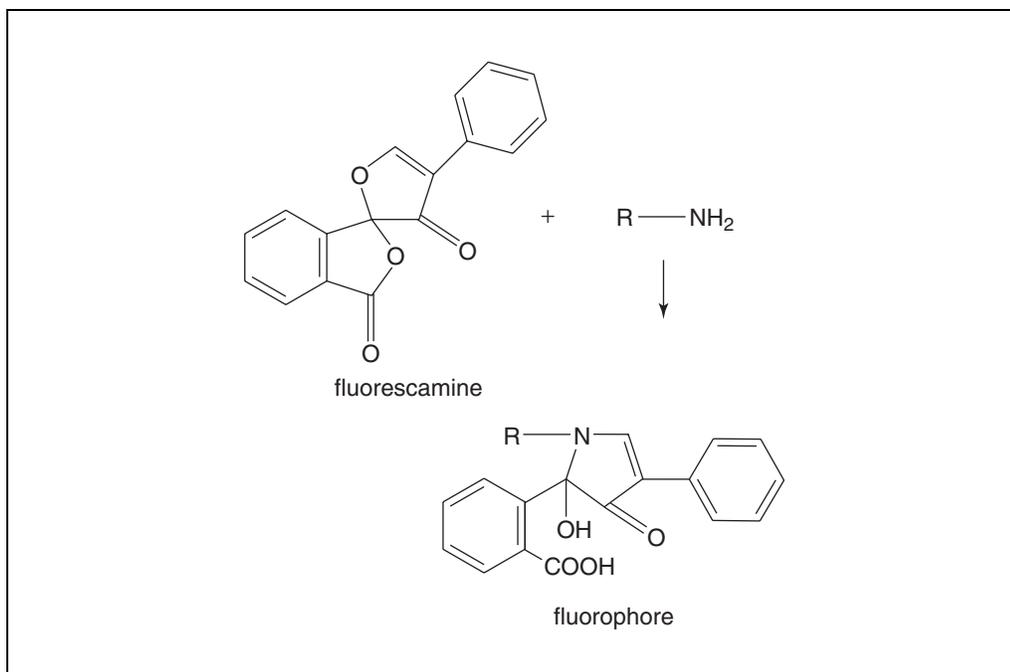

Figure B2.2.4 Fluorescamine reaction.

Evaluation of the Progress of Protein Hydrolysis

B2.2.6

Additional Materials (*also see Basic Protocol 1*)

   0.04 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*) *or* 0.04 M borate buffer, pH 9.7 (see recipe)
   0.3 mg/ml fluorescamine reagent in acetone
   Spectrofluorometer (excitation 390 nm; emission 475 nm)

1. Perform enzymatic hydrolysis of protein samples at a series of time points (see Basic Protocol 1, steps 1 to 5). Prepare a series of standards and a blank (see Basic Protocol 1, steps 6 and 7).

2. Set the spectrofluorometer to 390 nm excitation and 475 nm emission.

3. To a glass spectrofluorometer cuvette, add 0.20 ml of 0.04 M sodium phosphate buffer, pH 7 (for peptides) or borate buffer, pH 9.7 (for free amino acids).

   Usually, in food technology, the hydrolysis of a protein product is not intended to proceed to the production of free amino acids, because extended hydrolysis yields a product with poor functional properties. Therefore, it is important to know the amount of free amino acids generated by the hydrolysis. The TNBS reaction (see Alternate Protocol 1) allows a useful tool to quantify the amount of free amino acids.

4. Add 0.20 ml of 0.3 mg/ml fluorescamine and 1.0 ml of hydrolysis sample containing 5 to 50 nmol amino acid.

5. Measure fluorescence of the mixture at 475 nm.

   The resulting fluorescence is proportional to the amine concentration.

6. Create a calibration curve by plotting fluorescence versus concentration of the standards.

7. Determine the degree of hydrolysis (see Basic Protocol 1, steps 12 to 14).

FORMOL TITRATION TO DETERMINE DEGREE OF HYDROLYSIS

Amino acids react with formaldehyde, liberating one H^+ ion from the amino group, which is potentiometrically titrated with a sodium hydroxide solution. (Formol titration is also useful for measuring total free amino acids; for titration of acid tastants, see *UNIT G2.1*.) Amino groups of histidine do not react and those of proline and hydroxyproline react about 75%. Tertiary amines such as those in the guanidine group do not interfere with the reaction. Protein containing significant amount of Pro and hydroxyPro should be evaluated using other techniques.

$$H_2C=O + H_2N-CRH-COOH \rightarrow HC=N-CRH-COOH + H^+ + H_2O$$
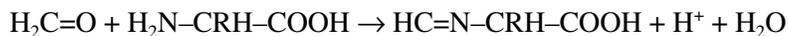
Additional Materials (*also see Basic Protocol 1*)

   0.25 N NaOH
   35% (w/v) formaldehyde, pH 8.1 (adjust pH with 10 M NaOH)

   13 × 100–mm glass test tubes
   10-ml buret or potentiometric titration apparatus

1. Perform enzymatic hydrolysis of protein samples at a series of time points (see Basic Protocol, steps 1 to 5) and prepare a sequence of standards and a blank (see Basic Protocol 1, steps 6 and 7). Scale up all samples, standards, and blanks to 2.5 ml in 13 × 100–mm glass test tubes.

2. Adjust pH in all tubes to 8.1 with 0.25 N NaOH.

Biochemical Compositional Analyses of Proteins

B2.2.7

Table B2.2.1 Calibration Factors ($1/\alpha$) for pH-Stat at Various Temperatures[a]

pH	Calibration factor				
	Temperature (pK for amino groups)				
	25°C	30°C	40°C	50°C	60°C
	(7.7)	(7.6)	(7.3)	(7.1)	(6.9)
6.5	—	—	—	5.00	3.50
7.0	—	5.00	3.00	2.27	1.79
7.5	2.59	2.27	1.63	1.40	1.25
8.0	1.50	1.40	1.20	1.13	1.08
8.5	1.16	1.13	1.06	1.04	1.03
9.0	1.05	1.04	1.02	1.01	1.01
9.5	1.02	1.01	1.01	1.00	1.00
10.0	1.01	1.00	1.00	1.00	1.00
10.5	1.00	1.00	1.00	1.00	1.00
11.0	1.00	1.00	1.00	1.00	1.00

[a]From Adler-Nissen (1986) with permission.

3. Add 1 ml of 35% formaldehyde, pH 8.1.

4. Incubate 1 min at room temperature and titrate with 0.25 N NaOH to pH 8.1. Record the volume of NaOH needed to reach the potentiometric endpoint.

   If more than 2 ml of 0.25 N NaOH is needed, repeat the assay using 1.5 ml formaldehyde.

5. Calculate the degree of hydrolysis (DH) of the substrate from the equation:

$$DH = B \times N_B \times 1.5 \times (1/M_P) \times (1/h_{tot}) \times 100\%$$

where B is the volume of NaOH used for maintain the pH at 8.1 value, N_B is the normality of the base, 1.5 is $1/\alpha$ (Table B2.2.1), and h_{tot} is 8.2 (for casein; Table B2.2.2).

Table B2.2.2 Content of Peptide Bonds for Various Food Proteins[a]

Protein material source	h_{tot} in meq/g ($N \times f_N$)
Casein	8.2
Whey protein concentrate	8.8
Meat	7.6
Hemoglobin	8.3
Gelatin	11.1
Fish protein concentrate	8.6
Soya proteins	7.8
Wheat gluten	8.3
Maize protein isolate	9.2

[a]From Adler-Nissen (1986) with permission.

PROTEIN HYDROLYSIS USING THE pH-STAT TECHNIQUE

The pH-stat technique monitors the course of a reaction in which each peptide bond is hydrolyzed by one or several enzymes. pH-stat evaluates the progress of hydrolysis by titrating the released amino groups with an alkaline solution. Enzymes work at constant pH and temperature during the entire process, so that no buffering is needed.

An automated pH-stat method gives a direct measurement of the percentage of hydrolyzed peptide bonds, the degree of hydrolysis (DH). The DH is calculated using the equation:

$$DH = B \times N_B \times 1/\alpha \times 1/M_P \times 1/h_{tot} \times 100\%$$

where B (ml) is the volume of base consumed, N_B is the normality of the base, $1/\alpha$ is the average degree of dissociation of the α-amino groups related with the pK of the amino groups at particular pH and temperature (Table B2.2.1), M_P (g) is the amount of the protein in the reaction mixture, and h_{tot} (meq/g) is the sum of the millimoles of individual amino acids per gram of protein associated with the source of protein used in the experiment (Table B2.2.2; Adler-Nissen, 1986).

As an example, this protocol describes how to perform the reaction using a casein substrate and a commercial proteolytic enzyme cocktail. The activity of the commercial four-enzyme cocktail is 35 U/ml, but should be tested prior to the experiment in order to determine the appropriate amount of enzyme needed for the reaction. The proteolytic activity assay (García-Carreño et al., 1994) is described below.

The pH-stat method can be modified and applied to a particular enzyme or substrate to assay any enzyme/substrate combination. The substrate can come from any source of protein such as poultry, milk, soybean, or fish processing byproducts. The amount of protein in the reaction should not exceed 8% (as calculated in Basic Protocol 1). The enzyme can be any alkaline endopeptidase such as Alcalase, trypsin, or chymotrypsin, and should be used in the proportions indicated in Basic Protocol 1. The selection of the appropriate enzyme depends on its efficiency and cost.

Materials

Four-enzyme cocktail (see recipe)
50 mM Tris·Cl, pH 8.0 at 25°C (*APPENDIX 2A*)
1% (w/v) azocasein in 50 mM Tris·Cl, pH 8.0 at 25°C
20% (w/v) trichloroacetic acid (TCA)
0.1 N NaOH
Casein from bovine milk powder, purified (Sigma)
Nitrogen

Spectrophotometer (366 nm)
Thermoregulable reaction vessel fitted with pH meter and temperature sensor
Water jacket or water bath set at 25°C
Autotitrator with 20-ml autoburet and recorder

Determine specific activity of enzyme cocktail

1. In six separate microcentrifuge tubes, mix 7 µl four-enzyme cocktail with 0.5 ml of 50 mM Tris·Cl, pH 8.0.

    A sample and a blank are each assayed in triplicate.

2. Add 0.5 ml of 20% TCA to the blank tubes only.

    For the blanks, 20% TCA is added before the substrate to inactivate the enzyme.

3. Initiate the reaction by adding 0.5 ml of 1% azocasein to all tubes.

4. Incubate 10 min and stop the reaction in the sample tubes by adding 0.5 ml of 20% TCA.

5. Microcentrifuge the reaction mixture 5 min at $6500 \times g$.

6. Record the absorbance at 366 nm (A_{366}).

7. Average the triplicate sample and blank values and subtract the mean of the blank from the mean of the sample.

8. Calculate the specific activity of the enzyme solution:

   activity (U/ml) = A_{366}/time (min)/volume (ml)

   For the four-enzyme cocktail used in this protocol, García-Carreño et al. (1994) reported an A_{366} of 0.49, giving an activity of 0.49/10 min/0.007 ml = 7 U/ml. The activity may vary depending on the stability of the enzymes under the given storage conditions.

Perform pH-stat assay

9. Calculate the total amount of raw substrate material (M_R) needed for the reaction:

   $$M_P = M \times S\%$$

   $$M_R = M_P/P\%$$

   In the first equation, M is the total mass of the hydrolysis reaction (10 g) and S% is the desired percent of substrate protein (casein) in the reaction (1%), giving the desired mass of substrate protein ($M_P = 0.1$ g). In the second equation, P% is the percent of substrate protein in the raw material, equal to the nitrogen content (N, determined by the Kjeldahl method; UNIT B1.2) times a conversion factor ($f_N = 6.24$). For casein with an N of 14.3%, $M_R = 0.1$ g/(14.3% × 6.24) = 0.11 g.

10. Calculate the amount of enzyme solution (V_{ES}) needed for the reaction.

    $$M_E = E/S \times M_P = 2\% \times 0.1 \text{ g} = 2 \text{ mg}$$

    $$V_{ES} = M_E/[\text{protein}] = 2 \text{ mg}/(2.8 \text{ mg/ml}) = 0.71 \text{ ml}$$

    The mass of enzyme (M_E) is based on a recommended enzyme/substrate ratio of 2%. The four-enzyme cocktail (see Reagents and Solutions) has 2.8 mg protein/ml, so a volume of 0.71 ml is needed. At 7 U/ml, this gives 5 U enzyme in the reaction.

11. Calculate the amount of water (M_W) needed for the reaction:

    $$M_W = M - M_{ES} - M_R = 10 \text{ g} - 0.71 \text{ g} - 0.11 \text{ g} = 9.18 \text{ g} = 9.18 \text{ ml}$$

12. Adjust the pH of substrate and enzyme solutions to 8.0 using 0.1 N NaOH.

13. Add 9.29 ml of casein substrate solution (total substrate and water calculated in steps 9 and 11, respectively) to a thermoregulable reaction vessel, insert pH and temperature sensors, and stir the solution using a magnetic stirrer. Set up an autotitrator with a 20-ml autoburet.

    Be careful not to stir too fast, as excess agitation can introduce CO_2 into the solution and change the pH.

14. Set the pH meter to 8.0, set the temperature sensor to 25°C, and charge the autoburet with 0.1 N NaOH solution.

15. Once the pH is constant, pump nitrogen over the reaction mixture.

16. Record the actual pH (should be 8.0).

17. Add 0.71 ml enzyme solution (total 10 ml reaction) and start the automatic titration.

The pH value of the reaction mixture goes down due to proteolytic activity, and is instantaneously maintained at 8.0 by the addition of NaOH solution.

18. Monitor titration by recording the volume of NaOH added every 5 min for 60 min or until the desired DH is reached.

19. Plot the progress of hydrolysis as a function of volume (in ml) of NaOH consumed versus time.

20. Calculate the degree of hydrolysis of the substrate from the equation:

$$DH = B \times N_B \times 1/\alpha \times 1/M_P \times 1/h_{tot} \times 100\%$$

where B (ml) is the volume of NaOH used to maintain pH at 8.0, N_B is the normality of the base, $1/\alpha$ is 1.5 (Table B2.2.1), and h_{tot} is 8.2 (Table B2.2.2).

21. Titrate a blank (same substrate with no enzyme) in the same manner.

No titrant solution should be consumed. Some substrates, while solubilizing, will reduce the pH of the solution and hence cause some consumption to occur; take this amount as zero.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Borate buffer, 0.04 M, pH 9.7

Dissolve 7.624 g sodium tetraborate decahydrate (reagent grade) in 400 ml deionized water. Adjust pH to 9.7 with 1 N NaOH. Bring to 500 ml with water. Store up 3 months at 4°C.

Citrate buffer, 200 mM

Dissolve 21 g citric acid monohydrate (reagent grade) in 400 ml water. Adjust pH to 5.0 with 1 N NaOH. Bring to 500 ml with water. Store up to 3 months at 4°C.

Four-enzyme cocktail

1.6 mg trypsin from porcine pancreas
3.1 mg chymotrypsin from bovine pancreas
1.3 mg aminopeptidase from porcine intestinal mucosa
8.0 mg pronase type XIV from *Streptomyces griceus*
Bring to 5 ml with distilled water
Store in 720–µl aliquots up to 2 months at −20°C

The final protein content of the solution is 2.8 mg/ml. The solution has 7 U/ml of specific activity, calculated using azocasein as substrate (see Basic Protocol 2, steps 1 to 8). All enzymes are available from Sigma.

Ninhydrin reagent

Dissolve 0.8 g $SnCl_2 \cdot 2H_2O$ in 500 ml 200 mM citrate buffer (see recipe) to give a 7 mM $SnCl_2$ solution. Dissolve 20 g crystalline ninhydrin in 500 ml 2-methoxyethanol (methyl cellosolve, Brenntag) to give a 4% (w/v) solution. Combine 500 ml $SnCl_2$ solution and 500 ml ninhydrin solution. Purge with nitrogen and store in dark bottles for up to one week at 4°C.

CAUTION: *Methyl cellosolve is toxic and ninhydrin stains everything it contacts.*

Biochemical Compositional Analyses of Proteins

B2.2.11

COMMENTARY

Background Information

Proteins are one of the most important ingredients in food production for both animals and humans. Besides having nutritional properties, protein contributes to the functional and organoleptic properties of food. The nutritional value of a protein depends on the total essential amino acid content. However, the availability of amino acids is conditioned by some protein attributes, mainly digestibility.

The most important feature affecting the functional and organoleptic properties of a protein is its surface structure. Surface structures affect the interaction of a protein with water or other proteins. By modifying the structure of the protein, particular functional and organoleptic properties are obtained. Functional properties of a protein are physicochemical characteristics that affect the processing and behavior of protein in food systems (Kinsella, 1976). These properties are related to the appearance, taste, texture, and nutritional value of a food system. Hydrolysis is one of the most important protein structure modification processes in the food industry. Proteins are hydrolyzed to a limited extent and in a controlled manner to improve the functional properties of a foodstuff.

In any quantitative work on protein hydrolysis, it is necessary to have a measure of the extent of the hydrolytic degradation. The measurement of the number of peptide bonds cleaved during a hydrolytic process is related to the activity of proteinolytic enzymes and the extent of hydrolysis. Various techniques that evaluate the progress of hydrolysis have been reported, such as the trichloroacetic acid (TCA) solubility index, which evaluates the percentage of nitrogen soluble in TCA after partial hydrolysis of the protein.

Degree of hydrolysis

A foodstuff (or other sample) obtained by hydrolysis of a protein material is called a *protein hydrolysate*. The *degree of hydrolysis* measures the percentage of peptide bonds hydrolyzed during protein hydrolysis (Adler-Nissen, 1976). An advantage of the DH concept is that for a given enzyme/substrate system the DH is independent of five variables: substrate concentration, enzyme/substrate ratio, pH, temperature, and time (Adler-Nissen, 1982).

The susceptibility of food protein to hydrolysis by digestive enzymes is called *digestibility* (*UNIT B2.1*). Proteins are hydrolyzed in industry by chemical or enzymatic means, just as they are in the digestive system. Chemical techniques for protein hydrolysis are harsh and extensive, and are generally only useful for amino acid composition analysis. Enzymatic proteolysis is highly specific in both the reactions it catalyzes and the products it yields. Enzymatic reactions can occur under mild conditions of temperature, pH, ionic strength, and pressure. In contrast to chemical hydrolysis, the nutritional value of the amino acids is maintained and functional properties are improved.

The degree of hydrolysis is calculated using hydrolysis equivalents (h), the number of peptide bonds cleaved during hydrolysis, expressed as eq/kg protein or meq/g protein. Hydrolysis equivalents are assayed by measuring the increase in free amino or free carboxyl groups that are produced by the hydrolysis of some protein. Several techniques have been described to assess the amount of amino and carboxyl groups before and after protein hydrolysis. The ninhydrin, TNBS, and fluorescamine reactions and the formol titration technique are used to evaluate the free amino group. The pH-stat technique is used to evaluate the free α-amino groups.

Sampling techniques

The ninhydrin, TNBS, and fluorescamine reactions, and formol titration are usually used to evaluate the time course of protein hydrolysis by periodically taking samples. The hydrolysis product (i.e., the free amino group) in each timed sample is treated with a chromogen or a fluorescent reagent and the product is evaluated by spectrophotometry. The result is proportional to the concentration of free amino groups. In the case of formol titration, reaction of the sample with formaldehyde liberates one H^+ ion from the amino group, which is potentiometrically titrated with a sodium hydroxide solution. Quantification of the number of amino groups gives the number of peptide bonds hydrolyzed and, hence, yields the hydrolysis equivalents, h. For time-course techniques, the DH is related to h by the equation $DH = (h/h_{tot}) \times 100\%$, where h_{tot} is the sum of the millimoles of individual amino acids per gram in the unhydrolyzed protein (Adler-Nissen, 1986).

Several assay techniques are available for quantitating amino groups. The ninhydrin technique is designated as Basic Protocol 1 because it is the most frequently used technique. The
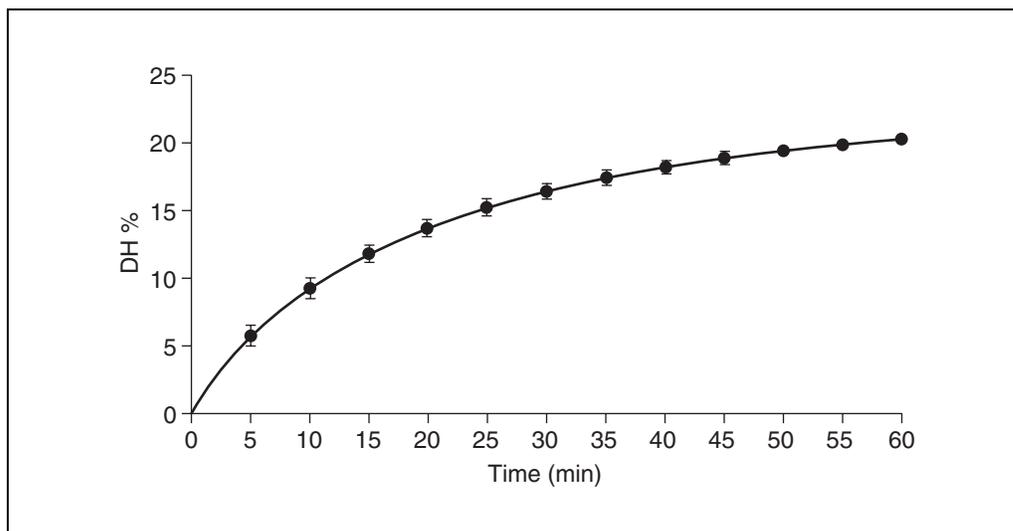
Figure B2.2.5 A typical graph of the progress of protein hydrolysis by the pH-stat technique.

trinitrobenzenesulfonic acid (TNBS) technique was accepted by many researchers for determining the DH of food protein hydrolysates because of its reproducibility. Nevertheless, it was difficult to apply to all food proteins, as some food proteins are difficult to disperse (e.g., partially insoluble proteins). Adler-Nissen (1979) modified this method by dispersing the protein in buffer containing SDS, using a less alkaline phosphate buffer instead of carbonates, and setting the reaction time to 1 hr. This version is presented in Alternate Protocol 1. The fluoroscamine technique (Alternate Protocol 2) is reliable and rapid to evaluate. Formol titration (Alternate Protocol 3) is an alternative when the laboratory does not have pH-stat equipment or a spectrophometer and spectrofluorometer. Comparison of results from more than one of the amino-detecting time-course assays allows for greater confidence.

Kinetic techniques

The pH-stat technique is a strategy used to evaluate the hydrolysis of protein by specific enzymes in a nonbuffered aqueous medium at alkaline pH. Each protein is composed of a specific number of amino acids, h_{tot}, which is expressed as equivalents of peptide bonds per kilogram (or milliequivalents per gram) of protein. The h_{tot} for different food proteins is given in Table B2.2.2.

In some experiments, specific information is needed about the equivalent of peptide bonds hydrolyzed during the reaction, in which case $h = (B \times 1/\alpha \times N_B)/M_P$, where α is the average degree of dissociation of the α-amino groups

liberated during the hydrolysis, as determined by the equation $\alpha = (10^{pH-pK})/(1 + 10^{pH-pK})$. It is important to remember that the pK is dependent on temperature. The value of $1/\alpha$ is the calibration factor of α for pH-stat and is shown in Table B2.2.1.

If necessary, the size of peptides generated during the experiment can be evaluated using a 10% to 18% gradient SDS-polyacrylamide gel (Laemmli, 1970; *UNIT B3.1*). It is very important to be sure that there is no residual enzymatic activity when the desired DH has been reached. To guarantee no residual enzyme activity, a useful technique is substrate SDS-PAGE (García-Carreño, 1993).

Critical Parameters and Troubleshooting

The ninhydrin method is affected by sucrose concentrations >10% in samples hydrolyzed with 6 N HCl. The entire procedure is carried out in disposable tubes. Substances such as glycerol, phenol, and mannitol do not affect the assay results.

Anticipated Results

A typical graph of the progress of protein hydrolysis by the pH-stat technique is shown in Figure B2.2.5. The pH-stat technique offers reliability, reproducibility, and simplicity. In addition, the technique has the advantage that no secondary reaction is needed. However, if the equipment needed to perform a pH-stat experiment is not available, the ninhydrin and TNBS techniques are good alternatives.

Biochemical Compositional Analyses of Proteins

B2.2.13

The progress of protein hydrolysis is currently evaluated by measuring the degree of hydrolysis at intervals. Because a limited controlled hydrolysis of a food protein yields products of particularly interesting functional and organoleptic properties, the DH concept simplifies the evaluation of the progress of the hydrolysis.

Regardless of which technique is used to measure the DH, at least five independent indices can be defined for any given enzyme/substrate system: substrate concentration, E/S ratio, pH, temperature, and time. The advantage of using the DH is that four of these variables (S, E/S, T, t) can be replaced by DH (for details, see Adler-Nissen, 1982).

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