Taken together, these data suggest that chemical treatment of blood for reclamation of protein is feasible at the industrial level. Industrial application of chemical coagulation techniques could eliminate or diminish the need for secondary treatment of blood wastewaters since chemical coagulation is capable of quantitative removal of protein in the primary step. We are currently studying other chemical procedures of blood protein removal.

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Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid

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An accurate, reproducible and generally applicable procedure for determining the degree of hydrolysis of food protein hydrolysates has been developed. The protein hydrolysate is dissolved/dispersed in hot 1% sodium dodecyl sulfate to a concentration of 0.25–2.5 × 10^{-3} amino equivalents/L. A sample solution (0.250 mL) is mixed with 2.00 mL of 0.2125 M sodium phosphate buffer (pH 8.2) and 2.00 mL of 0.10% trinitrobenzenesulfonic acid, followed by incubation in the dark for 60 min at 50 °C. The reaction is quenched by adding 4.00 mL of 0.100 N HCl, and the absorbance is read at 340 nm. A 1.500 mM L-leucine solution is used as the standard. Transformation of the measured leucine amino equivalents to degree of hydrolysis is carried out by means of a standard curve for each particular protein substrate.

Enzymatically hydrolyzed proteins possess functional properties, such as low viscosity, increased whipping ability, and high solubility, which make them advantageous for use in many food products. Recent experiments have indicated that in order to obtain desirable organoleptic and functional properties of soy protein hydrolysates, the hydrolysis must be carried out under strictly controlled conditions to a specified (generally low) degree of hydrolysis (DH) (Adler-Nissen, 1977; Adler-Nissen and Sejr Olsen, 1979). DH is defined as the percentage of peptide bonds cleaved (Adler-Nissen, 1976). Therefore, a need exists for a general method of determining DH of food protein hydrolysates, in particular for quality control. An obvious method to consider for this purpose is the trinitrobenzenesulfonic acid (TNBS) method, by which the concentration of primary amino groups in the hydrolysate can be determined.

Basically, this method is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines (Figure 1). The reaction takes place under slightly alkaline conditions and is terminated by lowering the pH. TNBS also reacts slowly with hydroxyl ions, whereby the blank reading increases; this increase is stimulated by light (Fields, 1971).

Since its introduction by Satake et al. (1960), the TNBS method has enjoyed a widespread use for the determination of free amino groups of proteins and protein hydrolysates. However, the presence of insoluble proteinaceous material in, e.g., the commercially used whipping agents based on hydrolyzed soy protein necessitates certain modifications of the various existing procedures described in the literature, as they seem to have been developed for soluble materials only. Also, although it is generally assumed that a linear relationship between the color intensity and the concentration of α-amino groups exists, we have

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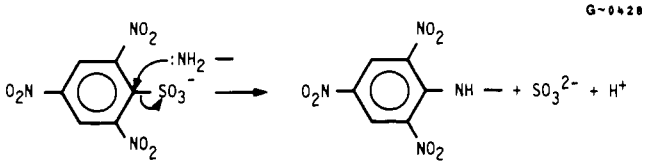

Figure 1. Reaction of TNBS with amino groups.

observed in this study that there is a considerable difference between the different proteins with respect to the actual value of the *slope* and *intercept* of this relationship. (The intercept, which is mainly due to the ε-amino groups, is of considerable and varying magnitude.) Thus, some way of standardizing the assay is needed. Finally, after some initial experiments using either a version of the original TNBS procedure (Satake et al., 1960) or a more rapid method described by Fields (1971), it was concluded that a more thorough study was needed in order to obtain a manual, accurate TNBS procedure for determining the DH of food protein hydrolysates in general. In particular, when using the above-mentioned methods, a high spreading of the results from repeated analysis on the same material was observed. This was soon ascribed to difficulties in dispersing the partially insoluble proteins and protein hydrolysates. With a view to these considerations it was decided in advance that the following features should be incorporated in the modified TNBS procedure.

(1) The sample should be dispersed in sodium dodecyl sulfate ($NaDodSO_4$) rather than buffer alone. Incorporation of $NaDodSO_4$ in the TNBS method has been reported by Habeeb (1966), who used this agent for denaturing the proteins in his samples. The use of mercaproethanol to prevent protein aggregation is ruled out because this agent reacts with TNBS (Kotaki et al., 1964).

(2) The buffer used in most versions of the TNBS method is bicarbonate (pH 8.5). As we considered the formation of CO_2 during the acidification step a nuisance, it was decided to use another buffer system. Borate, which was applied by, e.g., Fields (1971), does not seem to be recommendable if sugars are present (as they may be in our case), whereas phosphate is suitable (Burger, 1974). Also, phosphate allows the choice of less alkaline pH values than often used, which is advantageous in practice, as the blank reaction increases considerably if pH is above 8.5 (Satake et al., 1960).

(3) A reaction time of 1 h was considered optimal for the manual procedure, being neither so short as to make the color intensity sensitive to a few minutes of deviation on the reaction time, nor so long as to make it difficult to run the complete analysis in half a working day. Complete reaction should be achieved within the reaction time to ensure high reproducibility.

EXPERIMENTAL SECTION

Materials. Trinitrobenzenesulfonic acid dihydrate (analytical grade) and $NaDodSO_4$ were obtained from Sigma. All the other analytical chemicals were from Merck. The proteins (Kjeldahl nitrogen contents in parentheses) were soy protein isolate (Purina 500 E from Ralston Purina (14.1% N), casein according to Hammersten (Merck) (14.0% N), and gelatin (a commercial, low-Bloom, alkaline extracted gelatin from Extraco, Sweden; 15.7% N). The enzymes used for the hydrolyses were alcalase 6.0 FG and pancreatic trypsin Novo 6.0 S, both from Novo Industri A/S. The declared proteolytic activity of both enzyme preparations was 6 Anson units (AU)/g; for comparison the proteolytic activities of the crystalline enzymes are approximately 25 AU/g for Alcalase (Novo Industri 1978a) and approximately 20 AU/g for porcine trypsin (Novo Industri 1971).

The standard samples of soy protein hydrolysate, which were used in most of the experiments, were prepared according to the procedure described in the last subsection of the experimental section.

Reagents. The following reagents were used: 0.2125 M phosphate buffer [0.2125 M NaH_2PO_4 is added to 0.2125 M Na_2HPO_4 until pH is 8.20 ± 0.02 (the proportion of volumes is approximately 43:1000)], 0.1% TNBS solution [TNBS is dissolved in deionized water in a volumetric flask (100 or 150 mL) covered with aluminium foil; the solution must be prepared immediately before use], 0.100 N HCl, 1% $NaDodSO_4$, 1.500 mM leucine standard in 1% $NaDodSO_4$.

TNBS Reaction. Unless otherwise stated, the TNBS reaction was carried out as follows: 0.250 mL of a sample, containing between 0.25×10^{-3} and 2.5×10^{-3} amino equiv/L, is mixed in a test tube with 2.00 mL of phosphate buffer at pH 8.2. Two milliliters of 0.10% TNBS solution is added and the test tube is shaken and placed in a water bath at 50 ± 1 °C for 60 min. During incubation the test tubes and the water bath must be covered with aluminium foil because the blank reaction is accelerated by exposure to light. After the 60 min 4.00 mL of 0.100 N HCl is added to terminate the reaction, and the test tube is allowed to stand at room temperature (cooling below room temperature may cause turbidity because of the $NaDodSO_4$ present) for 30 min before the absorbance is read against water at 340 nm.

The reactions on the blank and the standard solutions are carried out by replacing the sample with 1% $NaDodSO_4$ and 1.500×10^{-3} M L-leucine in 1% $NaDodSO_4$, respectively. The absorbances of the blank and the standard are determined as the averages of six individual determinations.

The conditions during the reaction are as follows: buffer concentration = 0.10 M, TNBS concentration = 1.43×10^{-3} M, leucine concentration = 0.088×10^{-3} M, pH 8.2, temperature = 50 °C. pH after the HCl addition is 3.7–3.9; a pH below 3.5 will cause turbidity. The reaction can be considered pseudo-first-order with respect to the amino groups.

Definitions and Symbols. Only concepts and symbols which are used more than once in the test are included: a, intercept on the y axis of a regression line; AU, Anson units, a measure of proteolytic activity; b, slope of a regression line; DH, degree of hydrolysis, defined as the percentage of peptide bonds cleaved, thus $DH = (h/h_{tot}) \times 100\%$; E/S, enzyme–substrate ratio, based on protein substrate; A, absorbance; A_m, maximum absorbance estimated in kinetic experiments; h, hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein of α-amino groups formed during hydrolysis; h_{tot}, hydrolysis equivalent at complete hydrolysis to amino acids; h_{tot} is calculated by summing up the contents of the individual amino acids in 1 g of protein; k, first-order reaction constant; λ, spectrophotometric wavelength; s_0, standard deviation within the groups in grouped regression analysis; s_1, standard deviation around regression line in grouped regression analysis; $s(h)$, total standard deviation on h determinations; S, substrate concentration defined as concentration of Kjeldahl nitrogen multiplied by the appropriate factor. Sensitivity $\Delta A/\Delta$mequiv of $NH_2^- \times L^{-1}$ (change in A for a given change in concentration of amino equivalents.

Kinetic Experiments. The kinetic experiments were either carried out by varying the reaction time in the TNBS reaction described above or by using the following,

slightly more accurate procedure: a 5-mL sample was mixed with 40 mL of buffer and 40 mL of TNBS solution in an aluminium foil covered flask immersed in a water bath. All solutions had been heated to the temperature of the water bath prior to the experiments. At 5-min intervals a 4-mL sample was drawn and mixed with 4.00 mL of 0.094 HCl (this corresponds to mixing 4.25 mL with 4.00 mL of 0.1 N HCl as in the standard procedure). The absorbance was then immediately measured at 340 and 420 nm (the two absorption maxima for TNBS-substituted amino acids). A blank reaction was run simultaneously by the same procedure. The increase in the absorbance of the blank during the reaction follows a zero-order scheme, and the rate constant was determined by a linear regression analysis. The regression equation was then used to correct the sample readings for the contribution from the blank. The corrected sample readings were plotted as a function of time, and the first-order rate constant was determined direct from the plot by the method of least squares as described below.

Theoretically, the absorbance should follow the first-order relationship

$$A = A_m(1 - e^{-kt})$$

The sum of squared differences between the *n observed* absorbances, A_i (i = 1, n), and their theoretical values for the same $t = t_i$ (i = 1, n) is called z. Assuming a fixed, *estimated* value of k, $\hat{k}$, z becomes

$$z = \sum_1^n (A_i)^2 + A_m^2 \sum_1^n (1 - e^{-\hat{k}t_i})^2 - 2A_m \sum_1^n A_i(1 - e^{-\hat{k}t_i})$$

By differentiation of z with respect to A_m and setting $dz/dA_m = 0$ to obtain a minimum value of z, the best estimate of A_m, $\hat{A}_m$, is

$$\hat{A}_m = \frac{\sum_1^n A_i(1 - e^{-\hat{k}t_i})}{\sum_1^n (1 - e^{-\hat{k}t_i})^2}$$

and

$$z_{min} = \sum_1^n (A_i)^2 - \hat{A}_m \sum_1^n A_i(1 - e^{-\hat{k}t_i})$$

A different estimate of k would give different values of $\hat{A}_m$ and z_{min}. By trial-and-error a value of $\hat{k}$ is found, which gives the lowest value of z_{min}. The set of $\hat{k}$ and $\hat{A}_m$ thereby obtained is then taken as the best estimates of k and A_m, respectively.

The main advantages of the procedure above are that it assigns a constant variance to all A_i values, thereby reflecting the physical realities of the experimental design, and that A_m does not have to be independently estimated. The disadvantage is primarily that a confidence interval for $\hat{k}$ cannot be readily obtained.

The kinetics of the TNBS reaction was studied under a variety of conditions: pH 8.2 vs. pH 8.5, 40 °C vs. 50 °C, $NaDodSO_4$ vs. no $NaDodSO_4$. As samples were used 1.0 × 10^{-3} M L-leucine and a standard soy protein hydrolysate containing approximately 0.40 × 10^{-3} equiv/L of α-amino groups (the experiments were designed in such a way that an accurate knowledge of the concentration of amino groups was of no importance in the determination of the rate constant). Because the kinetic experiments serve only to optimize the TNBS reaction, a very rough determination of the average rate constant is sufficient, and it seemed justified from the results obtained to treat the protein hydrolysate as if all the amino groups (including the ε-amino groups) exhibit the same rate constant.

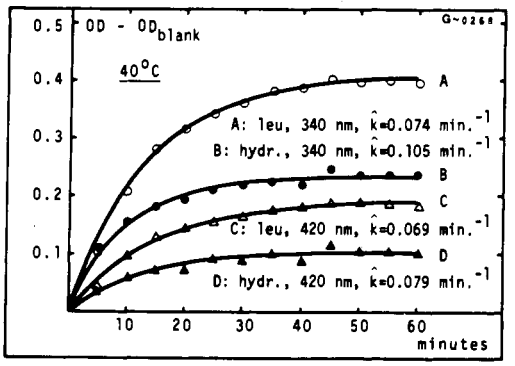

Figure 2. Kinetics of the pseudo-first-order reaction between NH_2 and TNBS at 40 °C and pH 8.2. The rate constants, $\hat{k}$, were determined by the method of least squares as described in the Experimental Section.

Protein Hydrolysis. The proteins were hydrolyzed in a thermostated, 1-L vessel equipped with stirrer and pH-stat equipment, including a recorder for the base (4 N NaOH) consumption (Adler-Nissen, 1977). All hydrolyses were carried out using the following hydrolysis parameters: S = 5.0 w/w % (N × 6.25) in 800 g of aqueous solution, E/S = 12 mAU/g (in one experiment: 30 mAU/g), pH 9.5 and 50 °C. Protein concentrations were later recalculated as Kjeldahl nitrogen multiplied by the appropriate conversion factors: soy protein, 6.25 (Smith and Circle, 1972); casein, 6.38 (Jones, 1931); gelatin, 5.55 (Jones, 1931).

During hydrolysis, 6-mL samples were drawn at specified times (typically after 5, 10, 20, 30, 40, 60, 80, 100, 120, and sometimes 160, 200, and 240 min of hydrolysis time) and quickly transferred to a test tube. From the test tube 2 × 2.00 mL were rapidly pipetted into two test tubes containing 10 mL of 1% $NaDodSO_4$ and kept at 75 °C by immersing in a water bath. The test tubes were shaken and maintained in the water bath for at least 15 min. It seems reasonable to assume that the enzymes were promptly inactivated by the treatment with the hot $NaDodSO_4$ solution, and the prolonged heat treatment mainly served to disperse the protein hydrolysate. After the heat treatment the contents of the test tubes were transferred quantitatively to 50-mL volumetric flasks and the volume was adjusted with 1% $NaDodSO_4$. The content of free amino groups, expressed as leucine amino equivalents, was assayed (single determination) by the TNBS reaction.

From the base consumption recorded, the hydrolysis equivalent (h) measured in milliequivalents/gram of protein could be calculated. The approximate pK value of the amino groups in polypeptides is 7.5 at 25 °C and the ionization enthalpy is in the range of 40–50 kJ/equiv of NH_2 (Steinhardt and Beychock, 1964). Using the Gibbs–Helmholtz equation (Castellan, 1964) on these data, the pK value at 50 °C can be calculated to approximately 7. This means that the α-NH_2 groups are fully titrated and the equivalents of peptide bonds cleaved are equal to the equivalents of base consumed. In these calculations, corrections were made for the gradual, slight decrease in the amount of hydrolysis mixture caused by the drawing of samples (Novo Industri, 1978b).

RESULTS AND DISCUSSION

Kinetic Experiments. Figure 2 shows the course of reaction between NH_2 and TNBS at 40 °C during the experiments carried out according to the modified procedure described in the Experimental Section. The solid curves are the reaction curves calculated from the estimated values of k and A_m (see the Experimental Section); it appears that the course of reaction is reasonably close

Table I. Determination of Rate Constants for the Pseudo-First-Order Reaction between NH_2 and TNBS in Excess[a]

sample	$NaDodSO_4$ in sample	temp, °C	pH	no. of analyses	λ, nm	k, min^{-1}	k_{av}, min^{-1}	blank, $\Delta A/min^{-1}$
L-leucine	–	50	8.2	6	340	0.115	0.133	see values below
	–			6	420	0.130		
	+			6	340	0.115		
	+			6	420	0.120		
	+			11	340	0.145		
	+			11	420	0.147		
	–		8.5	6	340	0.230	0.184	
	–			6	420	0.145		
	+			6	340	0.175		
	+			6	420	0.185		
	+	40	8.2	12	340	0.074	0.072	
	+			12	420	0.069		
soy protein hydrolysates	+	50	8.2	6	340	0.180	0.170	0.00195
	+			6	420	0.160		0.00100
	+			11	340	0.195		0.00172
	+			11	420	0.145		0.00090
	+		8.5	6	340	0.160	0.150	0.00217
	+			6	420	0.140		0.00114
	+	40	8.2	12	340	0.105	0.092	0.00132
	+			12	420	0.079		0.00090

[a] Concentration of TNBS, 1.43 mM; concentration of NH_2, ≤ 0.06 mM. Further details in the Experimental Section.

to an ideal first-order scheme.

The rate constants obtained in all the individual experiments are shown in Table I. A comparison between the rate constants obtained at 340 and 420 nm does not disclose any *systematic* effect of the choice of wavelength for the estimation of the rate constant, nor does the presence of $NaDodSO_4$ seem to have any systematic effect on the rate of reaction between TNBS and leucine. The values of the rate constants are therefore pooled to yield the average values shown in Table I. On the basis of these values it can be concluded that for both leucine and soy protein hydrolysate the reaction rate increases 1.85 times when the temperature is increased from 40 to 50 °C, a result which seems very reasonable indeed. It also appears that the reaction rate is higher for the hydrolysate than for the leucine standard.

With respect to the effect of pH, Table I shows that increasing the pH from pH 8.2 to 8.5 has a significantly positive effect on the reaction rate of leucine, whereas that of soy protein hydrolysate has hardly changed. This can be explained by the different pK values for the amino groups in the two systems. For leucine, pK can be calculated as approximately 9 at 50 °C, whereas the pK of the soy protein hydrolysate will be as low as 7, as explained previously. Because TNBS reacts only with the amino groups in their unprotonated state (Freedman and Radda, 1968), it is obvious that the pH increase from 8.2 to 8.5 will increase the concentration of reactive amino groups in the leucine system but not in the hydrolysate system.

For practical purposes it is satisfactory that the reaction is 99–99.5% complete, which is achieved when the product of k and the reaction time is above 5. For a reaction time of 60 min this requirement is fulfilled at 50 °C and pH 8.2, but not at 40 °C and pH 8.2. Increasing the pH to 8.5 may allow a slightly shorter reaction time; however, this also increases the zero-order rate constant for the blank reaction. Furthermore, the buffer capacity of the phosphate buffer is also considerably lower at that pH. The conditions of pH 8.2, 50 °C, and 60-min reaction time thus seem a reasonable compromise in the choice of reaction parameters.

Optimization of Postreaction Treatment. A series of experiments was carried out consisting of repeated assays of the standard leucine solution and the standard hydrolysate solution used in the kinetic experiments. All the assays were carried out according to the standard TNBS reaction, except that the normality of HCl (to vary the end pH) and the time interval between the HCl addition and the spectrophotometric reading were varied. The data obtained during this optimization were treated by simple analyses of variance, and it could be concluded that if the end pH was below neutral and the time interval was between 30 min and 3 h, no significant effect of these two parameters was observed. If the time interval was shortened to 15 min, it appeared, however, that the absorbances of the standard leucine were slightly higher. It was therefore concluded that a period of at least 30 min should elapse between the termination of the reaction and the spectrophotometric reading to ensure reproducible results.

Choice of Wavelength. In the choice between 340 and 420 nm, both sensitivity and reproducibility should be considered. The sensitivity is expressed as $A - A_{blank}$ for a given concentration of amino groups, and the reproducibility can be taken as the variation coefficient of $A - A_{blank}$ obtained by repeated assays of the same sample. Six repeated analyses on a number of leucine and soy protein hydrolysate standard samples covering a range of amino concentrations were therefore carried out. From these studies it could be concluded that the sensitivity for all samples was 1.5–1.8 times higher at 340 nm than at 420 nm. A comparison of the variation coefficients showed that these were fairly independent of the amino concentration and that for leucine the greatest reproducibility was obtained at 340 nm (3% vs. 7% at 420 nm on a *single* determination). For the hydrolysate the reproducibility was approximately the same for both wavelengths (4% on a *single* determination). Three hundred and forty nanometers thus seemed superior to 420 nm, and 340 nm was therefore chosen as the standard wavelength.

Demonstration of Linearity. Dilution series (eight concentrations) of a 4.0 mM leucine and of a soy protein hydrolyzate containing approximately 2.9 mequiv of NH_2^-/L were prepared and analyzed (triple determination). The 8 × 3 values of $A - A_{blank}$ (at 340 nm) were subjected to a grouped regression analysis. In both cases variance ratios close to unity were obtained and the assumption of linearity between NH_2 concentration and $A - A_{blank}$ could thus be sustained.

Table II. Results from Grouped Regression Analysis on the Data from the Hydrolysis Experiments[a]

regression parameter	soy protein hydrolysate I	II	III	casein hydrol. IV	V	gelatin hydrol., VI
$\bar{X}$ (mean of X)	0.5322	0.5488	0.8728	0.5920	0.3625	0.6325
$\bar{Y}$ (mean of Y)	0.8679	0.8595	1.1929	1.0067	0.7530	0.9604
b (slope)	1.0110	0.9968	0.9173	1.0148	1.0472	0.7957
s_1 (SD regr. line)	0.0263	0.0401	0.0302	0.0396	0.0233	0.0373
s_0 (residual SD)	0.0196	0.0289	0.0208	0.0409	0.0295	0.0174
F (variance ratio)	1.79	1.93	2.12	0.94	0.62	4.62*
SS_x (sum of $(X_i - \bar{X})^2$)	1.2373	1.3761	2.1017	1.5329	0.3865	2.4346
r (correl. coeff.)	0.998	0.996	0.998	0.997	0.994	0.996
n (no. of double det.)	9	9	9	9	11	11
signif. level for diff. between slopes[b]		0.950-0.975			0.70-0.80	
signif. level for diff. between s_1[c]			$\bar{s}_1 = 0.0333$ $P = 0.40$-0.50			
signif. level for diff. between s_0[c]			$s_0 = 0.0272$ $P = 0.90$-0.95			

[a] The following enzymes and enzyme-substrate ratios were applied: I and II, alcalase 12 mAU/g; IV, alcalase 12 mAU/g; V, trypsin 12 mAU/g; VI, alcalase 12 mAU/g. All hydrolyses were carried out at pH 9.5 and 50 °C. In the grouped regression analyses, X is equal to the value (in mequiv/g) of h (hydrolysis equivalents) obtained from the pH-stat and Y (two values of Y for each X) is equal to the measured amino content expressed in leucine mequiv/g. [b] Tested according to Hald (1951). [c] Barlett's test.

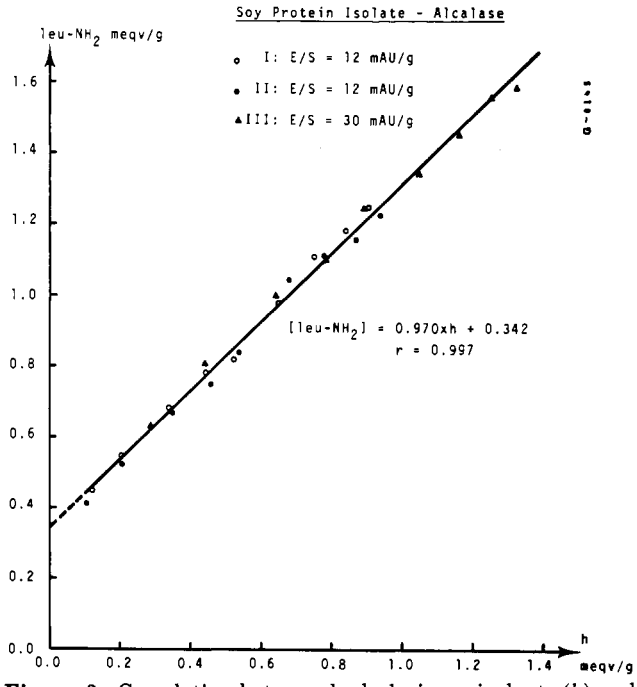

Figure 3. Correlation between hydrolysis equivalents (h) and leucine amino equivalents for the soy protein isolate–alcalase system.

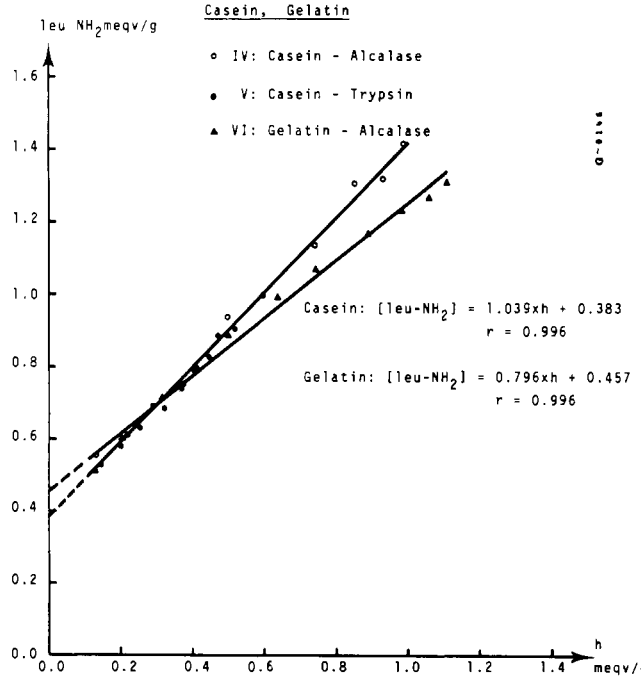

Figure 4. Correlation between hydrolysis equivalents (h) and leucine amino equivalents for casein and gelatin, respectively.

Demonstration of Quantitative Reaction. Dilution series of leucine and soy protein hydrolysate were assayed by the usual procedure. The assays were repeated and the reaction time was now prolonged from 60 to 90 min. Regression analyses of the A values vs. the concentration of amino groups yielded values of the sensitivity, expressed as the change in A for a given change in the concentration of amino groups. No significant change in the sensitivity for both leucine and soy protein hydrolysate was observed. This confirms the conclusion from the kinetic experiments, namely that a quantitative reaction between TNBS and the amino groups is achieved after a reaction time of 60 min. It is important to realize, however, that this result does not mean that the reaction time should not be carefully controlled. If the reaction times for the samples and the blanks differ more than a few minutes, a considerable error will be introduced because of the blank reaction.

Protein Hydrolysis Experiments. Six hydrolyses were carried out according to the procedure described in the Experimental Section. In the hydrolyses I, II, and III, soy isolate was hydrolyzed with alcalase (E/S = 12 mAU/g, 12 mAU/g, and 30 mAU/g, respectively). In IV and V, casein was hydrolyzed with alcalase and trypsin, respectively—both enzymes were applied in concentrations of 12 mAU/g. Finally, in VI, gelatin was hydrolyzed with Alcalase (12 mAU/g).

During each of the hydrolyses, samples were drawn at regular intervals. For each sample a value of h (hydrolysis equivalents), expressed as milliequivalents/gram, was obtained from the pH-stat, as described in the Experimental Section. In addition, the sample was assayed twice by the TNBS reaction. Thus, for each hydrolysis a set of data was obtained which could be subjected to a grouped regression analysis. The results of these regression analyses are summarized in Table II, and all the data are plotted in Figures 3 and 4.

It seems likely to expect that there will be no significant difference between the three regression lines, I, II, and III. The tests for significance (Hald, 1951) obviously rest on

Table III. Correlation between Hydrolysis Equivalents (h) and Leucine Amino Equivalents for Three Protein Hydrolysate

regression parameter		standard curves: Leu-NH_2 = $bh + a$ soy protein hydrolysate	casein hydrolysate	gelatin hydrolysate
$b \pm 1$ SD		0.970 ± 0.016	1.039 ± 0.022	0.796 ± 0.024
$a \pm$ SD		0.342 ± 0.012	0.383 ± 0.011	0.457 ± 0.017
Lys mequiv/g		0.42[b]	0.54[c]	0.32[d]
h_{tot} mequiv/g		7.75[e]	8.0[e]	11.1[e]
inverse curve	$1/b$	1.031	0.962	1.257
	$-a/b$	−0.352	−0.368	−0.574
total SD on h (4 determ.)	$h = 0.1$	0.019	0.018	0.028
	$h = 0.4$	0.021	0.020	0.027
	$h = 0.7$	0.024	0.024	0.030
	$h = 1.0$	0.029	0.031	0.036
	$h = 1.3$	0.035	0.038	0.044

[a] The standard curves were obtained by pooling the data from the individual hydrolyses (Table II). The total standard deviation on h is calculated under the assumption that the leucine amino equivalents are assayed by a quadruple determination using the standard procedure. The total standard deviation on h is composed of three independent sources of error: the leucine standard, the regression line for the standard curve, and the assay of the sample itself. [b] From amino acid analyses on the raw material, performed at the Institute of Protein Chemistry, Horsholm, Denmark, and at Bioteknisk Institut, Kolding, Denmark. [c] Value taken from Provansal et al. (1975), assuming 14.0% N in casein according to Hammarsten. [d] Value taken from Eastoe and Leach (1977). [e] Calculated from the amino acid compositions given in the references above.

the assumption, which is inherent in all regression analyses, that the uncertainty associated with the independent variable (in this case h) is zero. The standard deviation on h is in fact about 0.01 mequiv/g, which is one-third of the standard deviation on the dependent variable (Table II), and this is sufficient to explain the weak significance observed for hydrolysis VI. The picture is complicated by the fact that when different hydrolyses are compared, a systematic, relative error in h makes its contribution. This systematic error arises mainly from the uncertainty in establishing the substrate concentration (1.5% relative) and from the uncertainty in determining the absorbance of the standard leucine (1.8% relative). This adds a relative uncertainty to the *slope* in the order of 2–2.5%, whereby the weak significance for difference between the three slopes vanishes. Further calculations according to Hald (1951) shows that the three regression lines can indeed be regarded as expressing the same true relationship, and they are therefore pooled to yield the regression line shown in Figure 3.

Identical calculations as above show that there is no significant difference between the regression lines IV and V, although V has been hydrolyzed with trypsin instead of alcalase. These two regression lines are therefore pooled to give the regression line for casein in Figure 4.

Finally, it can be shown that the pooled regression lines for soy protein and casein are significantly different from each other both with respect to slope as well as intercept. These two lines are of course also different from the regression line for gelatin (VI), which has a markedly lower slope than the two other lines. Thus, it must be concluded that the slope and intercept for the regression lines, which describe the relationship between hydrolysis equivalents and leucine amino equivalents, assume particular values for each individual protein substrate. On the other hand, the results for casein hydrolysate, where two enzymes with completely different specificities (see, e.g., Hennrich, 1973) were applied, indicate that the *enzyme* has little or no influence on the slope and intercept. Table III gives the slope and intercept for the three different regression lines associated with hydrolysates of soy protein isolate, casein, and gelatin. As mentioned previously, the intercept is believed to be derived mainly from the presence of ϵ-NH_2 groups. However, the content of lysine expressed in milliequivalents/gram does not agree too well with the intercepts. For soy protein and casein hydrolysate the lysine content is 1.23 and 1.41 times higher, respectively. Now, it appears from the literature (Satake et al., 1960; Goldfarb, 1966) that there are considerable differences in the molar absorbance both between the individual amino acids as well as between amino acids and peptides, and the discrepancy may simply be caused by the ϵ-NH_2 groups in *peptides* having a molar absorbance of about three-fourths of that of L-leucine. Unfortunately, we have been unable to retrieve data on the molar absorbances of leucine and peptide-bound lysine measured under identical conditions, and this problem deserves further study. Another explanation for the low values of the intercepts could be that not all the lysine has been available for reaction.

The discrepancy between the intercept and the lysine content for *gelatin* hydrolysate may be due to the presence of small peptides and free amino acids in the raw material. In fact, it was found that 9.2% of the nitrogen was soluble in 0.8 M trichloroacetic acid, which supports this explanation.

From a practical point of view, however, the discussion above may be settled by the fact that, when the *intact* proteins were assayed by the TNBS procedure, the following results were obtained: soy protein isolate, 0.35 mequiv/g; casein, 0.43 mequiv/g; gelatin, 0.40 mequiv/g. These values lie reasonably close to the values obtained for the intercepts.

Accuracy of the Assay. The close linear relationship between the hydrolysis equivalent and the yield of the TNBS assay, expressed in leucine amino equivalents, means that the TNBS reaction can be used to determine the degree of hydrolysis (DH) of food protein hydrolysates by determining the hydrolysis equivalent (h) from the inverse standard curve.

The uncertainty on the determination of h is derived from three independent sources: the uncertainty on determining the absorbance on the sample itself (the uncertainty on the blank is small in comparison and can be neglected), the uncertainty on determining the absorbance of the leucine standard, and finally the uncertainty on the standard curve for that particular hydrolysate.

The first term (s_0 in Table II) appears to be fairly constant over a range of absorbance values (cf. "Choice of Wavelength") and independent of the type of hydrolysate. The second term can be shown to add an uncertainty to the absorbance which is proportional to the $A - A_{blank}$. The third term exhibits the usual curvilinear function of h. All three terms can be transformed to functions of h. It appears in this study that the three uncertainty terms are of the same order of magnitude. Very little is therefore gained by increasing the number of sample determinations

to, e.g., six or eight, and a quadruple determination should therefore generally be applied.

The total standard deviation on the three types of hydrolysate has been calculated as functions of h, and selected values are given in Table III. It appears that the differences in magnitude of $s(h)$ (standard deviation on h) are relatively small. The standard deviations shown correspond to a standard deviation on DH of 0.2–0.3% absolute, which should be satisfactory for all purposes (in this calculation the *systematic* error arising from the uncertainty on h_{tot} is neglected).

CONCLUSION

The present work has shown that an accurate, reproducible, and generally applicable determination of DH (the degree of hydrolysis) can be based on a modification of the TNBS reaction, as described in detail in the Experimental Section.

It is our experience from the use of the TNBS assay that now and then suddenly a large spreading of the results occur. This spreading can generally be traced back to inhomogeneities in the $NaDodSO_4$ sample solution. Certain protein and protein hydrolysate products are difficult to disperse in $NaDodSO_4$ without having been reduced by a disulfide reducing agent. Unfortunately, mercaptoethanol and other reducing agents containing sulfhydryl groups ruin the assay, as stated previously. Often it is possible, however, to disperse the protein without the use of mercaptoethanol by *homogenizing* the material in hot $NaDodSO_4$ and this procedure is therefore recommended.

Another potential source of error is deterioration of the leucine standard. Fresh standards should therefore be prepared regularly, and for accurate work it is recommended to use an additional standard as control, e.g., glycylglycine.

Except for the TNBS, the reagents used are relatively harmless. No particular safety precautions need to be taken except that gloves should be worn when making the TNBS solution.

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Determination of the Total Pepsin–Pancreatin Indigestible Content (Dietary Fiber) of Soybean Products, Wheat Bran, and Corn Bran

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Successive pepsin and pancreatin digestions were used to determine indigestible content (IDC) of various soybean products and cereal brans. IDC included insoluble material as well as solubilized carbohydrate and protein separated by ultrafiltration (molecular weight above 5000). Total IDC as percent of dry matter was: corn bran, 97; soybean hulls, 86; wheat bran, 52; whole soybean, 23; soy protein concentrate, 40; and defatted soy flakes, 16. The IDC values include 3–25% soluble material recovered by ultrafiltration. Chemical analyses of the insoluble nondigestible fraction from soybean hulls indicated a composition of 71% cellulose, 20% hemicellulose, 9% lignin plus ash. The percent protein digestibility was estimated as: whole soybean, 68; defatted soy flakes, 81; soybean hulls, 60; soy protein concentrate, 61; corn bran, 43; and wheat bran, 60. The large values for undigested protein in soy protein products were unexpected.

Recent reports on the nutritional significance of indigestible components of human food have stimulated new interest in the characterization of these components and their functionality, particularly the plant polysaccharides. Trowell (1977) discusses the significance of fiber in the human diet and defines dietary fiber as "the remnants of plant cells resistant to the alimentary enzymes of man". Other discussions on the definition and application of the term "dietary fiber" include those of Van Soest and Robertson (1977), Southgate (1977), and Meyer and Calloway (1977).

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