Peptidoglutaminase Deamidation of Proteins and Protein Hydrolysates for Improved Food Use[1]

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The limited deamidating ability of peptidoglutaminase (PGase) toward intact food proteins (0–6% deamidation) can be significantly enhanced by prior protein hydrolysis and altering protein conformation by such means as moist heat. PGase deamidation increases protein solubility and improves emulsifying and other physical properties under mildly acidic conditions. A batch reactor method was developed for the large-scale PGase deamidation of food proteins. Michaelis-Menten kinetics for industrial reactions (mixed zero- and first-order) were used for predicting the behavior of the reactor and for calculating enzyme dosage required to completely deamidate a given quantity of protein. Using such a reactor in the deamidation of food proteins or protein hydrolysates can lead to new food proteins with superior functional properties from less functional starting materials.

KEY WORDS: Enzymatic deamidation, functionality, peptidoglutaminase, protein emulsification and protein-modifying enzyme.

Many food proteins contain a large proportion of amide groups, which can be converted to acidic groups by deamidation. Deamidation of proteins can be carried out by chemical or enzymatic means. The enzymatic approach is preferred to the chemical approach for a variety of reasons. These include the speed of reaction, mild reaction conditions, possibility of catalyst recovery and, most important, substrate specificity. Surprisingly, enzymatic deamidation of food proteins has received little attention until recently. Motoki *et al.* (1) and Kato *et al.* (2,3) reported the use of transglutaminase and proteases to deamidate food proteins. Kikuchi *et al.* (4) obtained peptidoglutaminase (PGase) from the soil microorganism *Bacillus circulans,* that catalyzes the deamidation of the carboxyamide of glutamine, where glutamine is part of a small peptide. They fractionated PGase into two active fractions, which they termed PGase I and PGase II based on their separation on DEAE-Sephadex. Gill *et al.* (5) detected limited deamidating activity of PGase II toward casein and whey protein hydrolysates.

Hamada *et al.* (6) also investigated *Bacillus circulans* PGase for the deamidating of soy proteins. PGase readily hydrolyzed the γ-amide of glutamine residues in soy peptides, but its activity toward soy proteins was small (0.4–1.3% deamidation). Hamada and Marshall (7) found that heating increased PGase activity toward soy protein. They also investigated the combined effects of heat and proteolysis of soy protein on its deamidation by PGase. The present research is aimed at improving the functional properties, particularly solubility and emulsification, of food proteins under acidic conditions by PGase deamidation. The methods that have been previously used for PGase deamidation of proteins have been limited to the basic research level and may not be suitable for industrial application. Developing enzymatic processes for industrial scale-up of the deamidation of soy and other food proteins would make it possible for the food processor to use this procedure of protein modification. The objective of this research was to develop a large-scale deamidation process to produce enzymatically deamidated protein for food uses.

MATERIALS AND METHODS

Materials. Bacillus circulans culture (ATCC # 21590) was obtained from the American Type Culture Collection, Rockville, MD. CBZ-L-glutamine, egg albumin, wheat gluten, corn gluten, corn gluten hydrolysate, and an enzymatically hydrolyzed soy protein (Peptone type IV) were purchased from Sigma Chemical Co. (St. Louis, MO). Other enzymatic protein hydrolysates were obtained from Deltown Chemurgic Co. (Fraser, NY). *t*-BOC-L-glutaminyl-L-proline was purchased from Peptides International, Louisville, KY, and the BCA (bicinchoninic acid) protein assay reagent from Pierce Chemical Co. (Rockford, IL). Other chemicals were of the highest purity obtainable.

Preparation of PGase cell extract. B. circulans cells were grown in a medium composed of 1.0% polypeptone, 0.5% lactose, 0.025% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.025% KH_2PO_4 and 0.17% $Na_2HPO_4 \cdot 12H_2O$, at 30°C for 17 hr. Then the cells were harvested and the PGase was extracted with 0.01 M phosphate buffer, pH 8.0, as previously described (6).

PGase assay. Cell extract was evaluated for PGase I and II activities in 0.05M phosphate buffer, pH 7.0 at 30°C with CBZ-L-glutamine and *t*-BOC-L-glutaminyl-L-proline as substrates, respectively. Ammonia was determined with a Model 95-10 ammonia electrode (Orion Research Inc., Cambridge, MA). One unit of enzyme activity frees one micromole of ammonia from the substrate in 0.05 M phosphate buffer, pH 7.0–8.0 after 1 hr incubation at 30°C.

PGase deamidation of proteins or protein hydrolysates. The protein or protein hydrolysate was solubilized in 0.02M NaOH to a concentration of about 5% total protein by stirring at 400 rpm and 25°C for 1 hr. Reaction mixtures containing 25 and 20 units of PGase I and II and 1.2 mmoles protein amides in 0.05 M phosphate buffer, pH 7.5, were incubated at 30°C for 4 hr with stirring at 400 rpm. The ammonia released was determined with the ammonia electrode. The extent of protein deamidation was calculated as the ratio of ammonia released enzymatically to the total amide content.

Kinetic constants. One milliliter enzyme preparation containing 0.25 mg protein was added to 0.5–5.0 mL of 2% peptone type IV in 0.05M phosphate buffer, pH 7.0.

[1]Part of a symposium entitled "Preparation and Processing of Chemically-Physically Modified Proteins" held April 25, 1990, Session II at the 81st Annual Meeting of AOCS in Baltimore, MD.

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Reaction mixtures were incubated at 30°C for 30 min, then the ammonia content of the samples was determined. The K_m and V_{max} values of PGase with peptone type IV in 0.05 M phosphate buffer, pH 7.0, were measured at 30°C by determining the change in the rate of PGase reaction (v) with increasing substrate concentration [S]. The 1/v was plotted against 1/[S] to obtain a straight line (Lineweaver-Burk plot) with the values of $1/K_m$ and $1/V_{max}$ taken from the intercepts with the 1/[S] and 1/v axes, respectively.

Experimental reaction time course. Reactions were initiated by adding 50 mL of deionized water containing 40 mg PGase to 850 mL peptone solution in 0.05 M buffer, pH 7.0, containing 15.0 g peptone type IV. The reaction mixture was incubated at 30°C, and a 10-mL sample (in duplicates) was taken at zero time and every 30 min for ammonia analysis. The progress of the deamidation reaction as a function of time was plotted.

Calculated reaction time course. The Michaelis-Menten equation and the Michaelis-Menten equation integrated for mixed zero- and first-order kinetics (8) were used to calculate the conversion of the peptone substrate in 0.05M buffer, pH 7.0 at 30°C, as a function of time.

Chemical analyses. Protein contents for proteins or protein hydrolysates were measured by the Kjeldahl method and for PGase by the BCA method of Smith *et al.* (9). The free ammonia and amide contents of the protein or protein hydrolysate were measured according to methods of Wilcox (10). The ammonia released by the protein or protein hydrolysate after amide hydrolysis with 2 N HCl at 100°C for 4 hr was measured with the ammonia electrode. Corrections were made to account for the free ammonia in proteins or protein hydrolysates.

RESULTS AND DISCUSSION

PGase deamidation of food proteins and protein hydrolysates. PGase deamidation of selected food proteins and protein hydrolysates was carried out with laboratory-prepared and commercial samples (Table 1). All native proteins examined contained insignificant amounts of free ammonia (0.015–0.025 mmoles/g protein). No significant amide hydrolysis occurred during the preparation of soy protein hydrolysates by means of an alkaline protease, heat treatment or a combination of both (7). Some of the commercial enzymatic protein hydrolysates, however, contained considerable amounts of free ammonia, which indicated protein deamidation had occurred during their preparation. In these samples, free ammonia ranged from 0.032 to 0.250 mmoles ammonia/g protein. Considering the total amide content in these hydrolysates (Table 1), these levels of ammonia were equivalent to 0.5–29.4% of the total amide in the hydrolysates.

Table 1 gives the percentage PGase deamidation of proteins and protein hydrolysates. The enzyme activity toward intact protein was found to be small (0 to 6% deamidation). The PGase deamidation of hydrolysates was extensive, even for protein hydrolysates such as casein hydrolysate, which showed extensive amide hydrolysis prior to the PGase treatment. The enzyme readily hydrolyzed the amide of glutamine residues in peptides and protein hydrolysates. Therefore, the use of prior proteolysis substantially enhanced the deamidation of protein by PGase. This is in agreement with our initial report on the potential use of PGase in soy protein deamidation. Hamada and Marshall (7) investigated the potential of other means besides hydrolysis to increase PGase de-

TABLE 1

Analysis of PGase Deamidation of Food Proteins and Protein Hydrolysates

Protein or protein hydrolysate	N.F.[a]	Protein[b] (%)	Free NH_3 mmoles/g protein	Total amide mmoles/g protein	% Deamidation	
					Non-PGase[c]	PGase[d]
Water extract of soy flakes	6.25	56.9	0.026	1.00	0.0	6.4 (2)
Soy peptone (type IV)	6.25	58.5	0.032	0.68	0.5	56.0 (7)
Papainic soy hydrolysate	6.25	80.6	0.036	0.90	1.0	14.5 (3)
Wheat gluten	5.70	73.0	0.018	2.50	0.0	1.3 (1)
Corn gluten	5.60	56.6	0.032	1.23	0.0	2.8 (1)
Corn gluten hydrolysate	5.60	54.9	0.174	1.22	9.1	30.9 (5)
Rice protein hydrolysate	5.95	82.3	0.163	1.38	9.1	13.7 (3)
Egg albumin	6.25	82.5	0.017	0.86	0.0	0.0 (1)
Papainic egg albumin hydrolysate	6.25	76.3	0.036	0.65	2.9	39.6 (6)
Lactalbumin hydrolysate (DC-A)	6.38	78.5	0.121	0.71	11.2	39.0 (6)
Lactalbumin hydrolysate (DC-K)	6.38	61.9	0.146	0.85	11.5	37.4 (6)
Pancreatinic casein hydrolysate	6.38	79.8	0.099	0.88	8.6	30.4 (5)
Casein hydrolysate (DC-L)	6.38	70.8	0.249	1.12	17.2	27.2 (4)
Casein hydrolysate (DC-N)	6.38	79.1	0.450	0.95	29.4	26.3 (4)

[a]N.F., nitrogen factor.

[b]% Protein, % N × N.F.

[c]Calculated from values of free NH_3 and total amide of the protein.

[d]Multiple range test indicated that seven homogeneous groups were possible in which there were no significant differences between means constituting each group. In parentheses is the number of the group where the sample belongs.

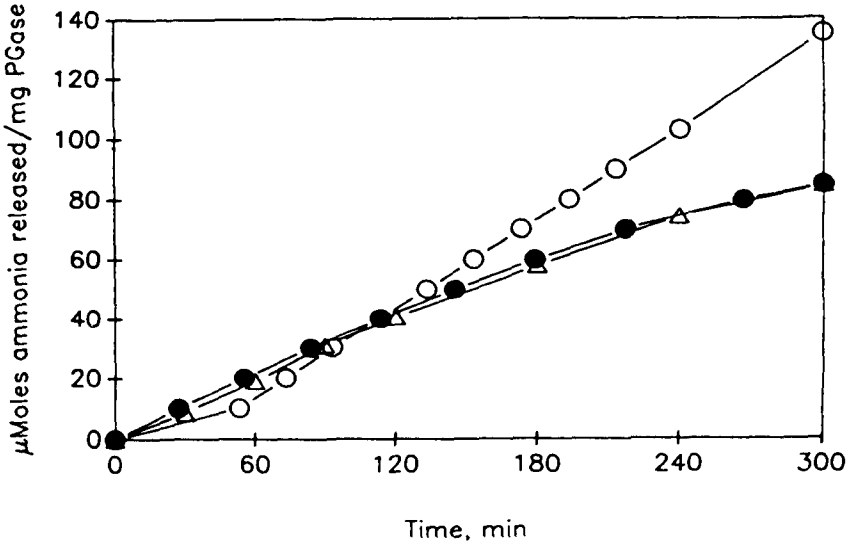

FIG. 1. Progress of Peptone IV deamidation by PGase: A (○) calculated from Michaelis-Menten equation 1; B (●) predicted from Michaelis-Menten equation for mixed 0- and 1st-order kinetics (equation 2); C (△) measured experimentally.

amidation of soy protein. They showed that heat treatment combined with proteolysis significantly enhanced soy protein deamidation by PGase, up to 27 times for 20% DH hydrolysates. Hamada and Marshall (11) found that small levels of deamidation (6–16%) increased soy protein solubility and substantially enhanced emulsifying activity under mildly acidic conditions (pH 4–6). Emulsion stability and foaming power of soy protein were also improved by PGase deamidation.

Scale-up production of deamidated proteins and protein hydrolysates. A batch-reactor method was developed for large-scale deamidation of food proteins with PGase. In this method, Michaelis-Menten kinetics were used to predict the potential activity and performance of the enzyme in reactors in order to control the reaction at optimum conditions. To study the enzyme kinetics, the PGase activity toward a commercial soy protein hydrolysate, Peptone type IV, was determined at various substrate levels in 0.05 M phosphate buffer, pH 7.0 at 30°C. The Lineweaver-Burk plot gave K_m value of 1.67×10^{-4} M and V_{max} value of 0.47 micromoles/min/mg for Peptone type IV. Two Michaelis-Menten equations were used in an attempt to predict the time course of PGase deamidation of the soy protein hydrolysate.

$$v = V_{max}\,[S]/[S] + K_m \qquad [1]$$

where v is the initial rate of enzyme-catalyzed reaction and [S] is substrate concentration.

$$t = S_0\,X + K_m \bullet \ln\,[1/(1-X)]/V_{max} \qquad [2]$$

The symbol t is reaction time; S_0 is initial substrate concentration; and X is the conversion rate, which is defined as:

$$X = (S_0 - S)/S_0 \qquad [3]$$

where S is substrate concentration at time t.

Equation 1 is the original Michaelis-Menten equation for zero-order reactions. Equation 2 is an integrated form of equation 1 suggested by Fullbrook (8) for mixed zero- and first-order reactions. One gram Peptone IV protein (N × 6.25) contained 0.67 mmoles amides, of which a maximum of 58.6% (mostly from glutamine residues) could be converted by PGase. Accordingly, in solving equation 2, hydrolysis of 0.40 mmoles ammonia from 1 g peptone protein was considered to be equivalent to 100% conversion or X value of 1.00 (equation 3).

The reaction time course was also determined experimentally by using 40 mg PGase and 15 g peptone in 0.05 M buffer, pH 7.0, at 30°C. A comparison of the reaction progress measured experimentally to reaction progress calculated from equations (1) and (2) is shown in Figure 1. As would be expected, the use of equation 1 in calculating the reaction progress yielded a straight line that was different from the experimental progress curve after 2 hr of reaction (curve A in Fig. 1). The reason is that the reaction velocity in the Michaelis-Menten equation is the velocity when the product concentration tends toward zero. But in a progress curve of a typical industrial reaction, which proceeds for long periods of time, the initial velocity decreases as the reaction proceeds. Because of the lowering of the average substrate concentration during such reactions, first- or mixed first- and zero-order kinetics, rather than zero-order kinetics are applied, such that the rate of reaction depends directly on the prevailing substrate concentration (8). As indicated in Figure 1, data from solving equation 2 to predict the time course of reaction (B) closely matched that measured experimentally (C). Accordingly, data obtained by equation 2 accurately demonstrate the time course of the reaction. Therefore, this equation is particularly good in providing the basis for modeling and optimizing configuration of deamidation reactors, and for determining the best

operating conditions to maximize productivity and to achieve complete amide hydrolysis.

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[Received November 27, 1990; accepted April 9, 1991]