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Note

Analysis of glutamine, glutamic acid and pyroglutamic acid in protein hydrolysates by high-performance liquid chromatography

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Because of its unstable nature, glutamine is normally analyzed after being deamidated to the more stable glutamic acid. However, both glutamine and glutamic acid could be easily converted to pyroglutamic acid (5-oxoproline, 2-pyrrolidone-5-carboxylic acid) in solution or in the dry state[1–3]. The formation of pyroglutamic acid causes more complications to the already difficult task of recovering glutamine from protein hydrolysis and estimating glutamine and glutamic acid in protein hydrolysates. Conventional methods for protein composition analysis, which use acid hydrolysis, analyze glutamine, glutamic acid and pyroglutamic acid in terms of Glx (the sum of the three amino acids). Special methods, which use enzymatic hydrolysis, analyze glutamine and glutamic acid individually but fail to recognize the possible discrepancy in glutamine and glutamic acid due to the formation of pyroglutamic acid. In order to deal with these problems, it is pertinent that methods be developed so that glutamine, glutamic acid and pyroglutamic acid in hydrolysates can be measured accurately. Reports on methods for amino acid analysis are numerous; however, few of them specialize in the analysis of glutamine and glutamic acid, and fewer still are those dealing with pyroglutamic acid. The analysis of glutamine (and asparagine) can be achieved in a modification of the conventional amino acid analysis by the use of lithium citrate buffer on ion-exchange resin[4,5], and the pyroglutamic acid in cosmetic products has been analyzed by the elution with sodium sulfate buffer on a TSK gel column[6]. In an earlier publication, we reported the use of high-performance liquid chromatography (HPLC) in the analysis of glutamine and asparagine[7]. In this study we (a) modified our earlier method for the analysis of glutamine and glutamic acid and developed a method using reversed-phase HPLC for the analysis of pyroglutamic acid, (b) analyzed protein hydrolysates in the presence of glutamine, glutamic acid and pyroglutamic acid before and after the deamidation of amide groups, and (c) investigated the formation of pyroglutamic acid and the stability of glutamine, glutamic acid and pyroglutamic acid.

EXPERIMENTAL

Apparatus

Analyses were performed on a Beckman Model 324 gradient liquid chromatograph equipped with a Beckman Model 421 microprocessor-controller, a Beckman

165 variable-wavelength UV detector and a Kratos Model FS970 liquid chromatographic fluorometer. The UV detector was set at 200 nm with a range of 0.1 a.u.f.s. The fluorometer settings were: excitation at 330 nm, emission measured with a 418-nm cut-off filter, time constant of 6 sec, and sensitivity at 5.8 units.

A Whatman Partisil PXS ODS column (250 × 4.6 mm I.D.; particle size, 10 μm) was used for chromatographic separations; the system also included a Whatman precolumn (250 × 4.6 mm I.D.) packed with 37–53 μm silica and a Whatman guard column (70 × 4.6 mm I.D.) packed with CO:PELL ODS sorbent (particle size, 30–38 μm). Chromatographic peaks were recorded on a Beckman Model BD-41 recorder, and integrated by analog-to-digital converters in a Hewlett-Packard 3345B laboratory automation system. Isocratic elution was used, unless otherwise indicated, and the flow-rate was 1.0 ml/min. The elution of glutamine and glutamic acid with sodium acetate buffer and acetonitrile was complete in 25 min, and the elution of pyroglutamic acid with 0.1% phosphoric acid was complete in 15 min. Elution of protein hydrolysates was programmed at the end of the run to 100% acetonitrile to wash off the remaining amino acids and followed by changing back to the original solvent and then equilibrating for at least 15 min before conducting another injection.

Reagents and solutions

Amino acids, 2-mercaptoethanol (ME), *o*-phthalaldehyde (OPA), casein, casein enzymatic hydrolysate, pronase E and proteinase K were purchased from Sigma. Acetonitrile was distilled-in-glass grade (Burdick and Jackson) and used without further treatment. High-purity water was obtained from a Milli-Q water purifier (Millipore). The buffers were prepared by mixing different ratios of stock solutions of 0.2 *M* acetic acid and 0.2 *M* sodium acetate to obtain various pH values and then diluting with water to desired concentrations. The eluting solvents were prepared by adding acetonitrile to acetate buffers, degassed for 30 min in an ultrasonic bath, and filtered through a 0.45-μm Millipore filter.

The OPA–ME derivatizing solution was prepared as follows: OPA (250 mg) was dissolved in methanol (6.3 ml). ME (250 μl) and 0.4 *M* potassium borate buffer (pH 10.5, 56 ml) were then added. The mixture was flushed with nitrogen and stored in the refrigerator. ME (100 μl) was added each day to help maintain the reagent strength.

Enzymatic protein hydrolysis

To a solution of 40 mg protein in 2.0 ml 0.1 *M* sodium hydroxide solution was added 5.0 ml 0.033 *M* borate buffer (pH 8.3), 1.0 mg proteinase K and 0.2 ml dialyzed pronase E (about 1 mg). The mixture was incubated at 37°C for 24 h and the hydrolysate solution filtered through a 0.45-μm Millipore filter.

OPA–ME derivatization

To a solution of 100 nmol amino acid or 1.0 μg protein hydrolysate in 0.3 ml water were added 0.2 ml OPA–ME solution. After 1 min at room temperature, 0.5 ml 0.1 *M* potassium phosphate (pH 4.0) was added, followed by 3.0 ml methanol. The solution was mixed, filtered, and injected (5 μl) onto the column.

RESULTS AND DISCUSSION

Analysis of glutamine and glutamic acid

Glutamine and glutamic acid are among the group of amino acids of which the OPA derivatives display the least affinity to a reversed-phase column. In elutions with media consisting of sodium acetate buffer and acetonitrile, the only OPA-amino acids that may be eluted ahead of OPA-glutamine and OPA-glutamic acid are the OPA derivatives of serine, histidine, asparagine, and aspartic acid. For the analysis of glutamine and glutamic acid, the time required can be short, and possible interferences from other amino acids are minimal. A simple isocratic elution is most suitable for our purpose, and resolutions of the elution peaks can be achieved by a control of the buffer pH. The effect of pH on retention time is shown in Fig. 1. With the increase of pH, retention time generally decreases for all OPA derivatives, more drastically so for OPA-glutamic acid and OPA-aspartic acid. Consequently, the elution peaks of OPA-glutamic acid and OPA-aspartic acid are most likely to interfere or coelute with one peak or another along the line of decreasing retention time as pH increases. For instance, at pH 4.20, OPA-aspartic acid coelutes with OPA-asparagine and OPA-glutamic acid with OPA-histidine (Fig. 2A). Higher buffer pH values are generally desirable because elution peaks at these pH values are sharper

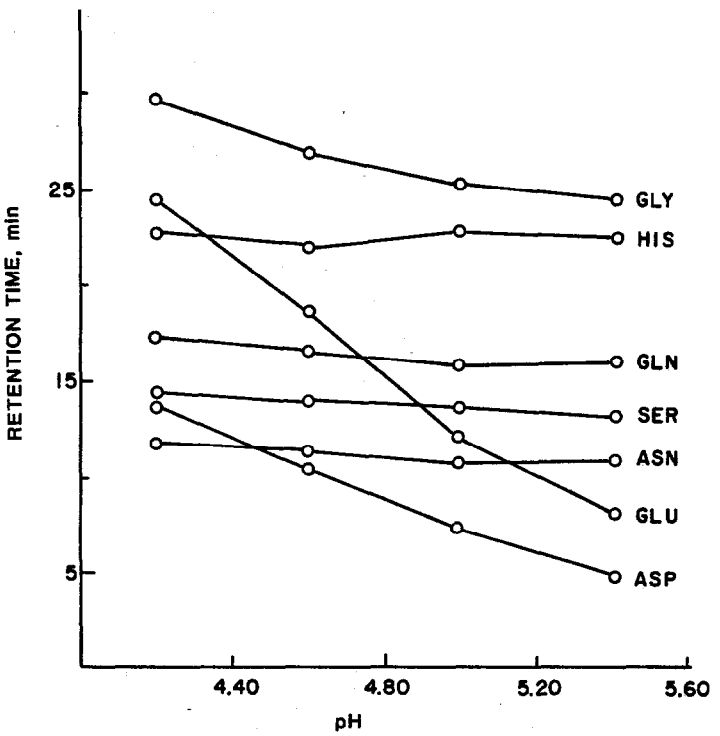

Fig. 1. Influence of pH on the retention time of OPA-ME amino acid derivatives. Retention times were obtained by isocratic elutions with solvents consisting of acetonitrile–0.04 *M* sodium acetate buffer, pH 4.20–5.40 (13.6:86.4). GLY = Glycine; HIS = histidine; GLN = glutamine; SER = serine; ASN = asparagine; GLU = glutamic acid; ASP = aspartic acid.

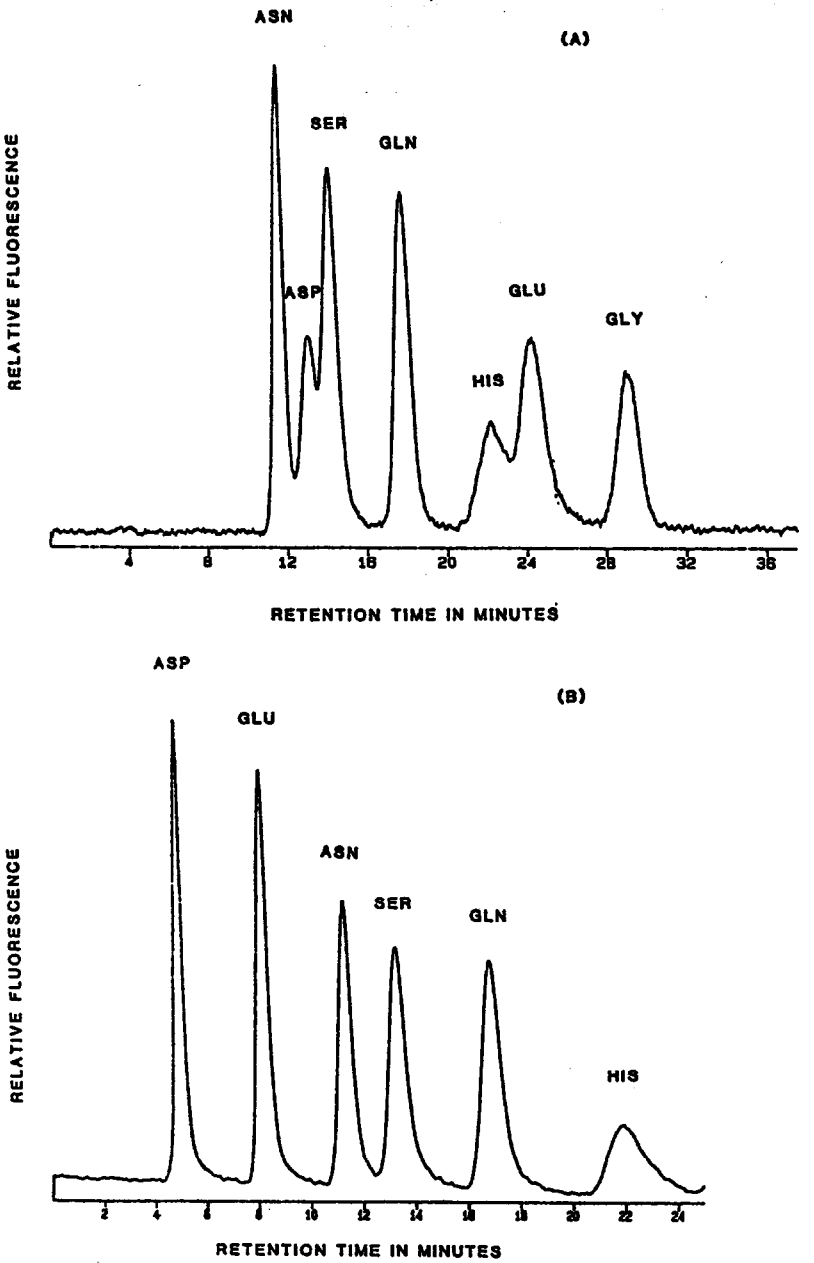

Fig. 2. Elution profiles of amino acid standards (125 pmol each) derivatized by the reaction with OPA–ME. The mobile phase consisted of acetonitrile–0.04 *M* sodium acetate buffer (13.6:86.4). For (A), the buffer pH = 4.20; for (B), the buffer pH = 5.40.

and more consistent. At high enough pH values, the two interfering glutamic acid and aspartic acid derivatives move ahead of all other elution peaks, making the separations that much easier. As can be seen in Figs. 1 and 2B, baseline separations are

achieved at pH 5.40, not only for glutamine and glutamic acid but also for aspartic acid, asparagine, and serine. Linearity of response for glutamine and glutamic acid was established in the concentration range of 50–250 pmol, and the quantitation of these two amino acids can be readily achieved. The solvent system which consists of sodium acetate buffer at pH 5.40 was therefore chosen for the following investigations. It should be noted that elution profiles may vary slightly from column to column which could be significant for a particular purpose such as the analysis of glutamine and glutamic acid. To ensure a successful analysis, a preliminary pH study such as shown in Fig. 1 should be conducted for each column. In our earlier investigations[7], we used a short (150 mm) Beckman ODS column, and baseline separations of glutamine, asparagine, glutamic acid, and aspartic acid were achieved at pH 4.85. We used a new Whatman ODS column (250 mm) in the present study and, by raising the buffer pH to 5.40, this longer column provided a different elution profile for the same group of amino acids and apparently better and more consistent separations.

Glutamine and glutamic acid in protein hydrolysates

The method described above proved to be very effective for the analysis of glutamine and glutamic acid in protein hydrolysates. Profiles in Fig. 3 are of the OPA derivatives of two casein hydrolysates. Fig. 3A is of casein which has been hydrolyzed by enzymes in our laboratory, and Fig. 3B is of an enzymatic casein hydrolysate obtained from a commercial source. Note that the glutamine peak in Fig. 3B is missing whereas its glutamic acid peak is much bigger than the corresponding peak in Fig. 3A. It is logical to assume that the loss of glutamine and the gain in glutamic acid are the result of deamidation which may have occurred during or after the hydrolysis. However, further investigations show that sometimes the loss in glutamine cannot fully account for the gain in glutamic acid. When our laboratory casein hydrolysate was completely deamidated by 2 *M* hydrochloric acid at 100°C for 2 h, the loss in glutamine could only account for 50% of the excess of glutamic acid. In the case of the commercial casein hydrolysate, the amount of glutamic acid increased by 3.5 times after the acid treatment. Note also that asparagine was present in both our laboratory hydrolysate and the commercial product, and that the loss of asparagine was always equivalent to the gain in aspartic acid in an acid deamination.

Analysis of pyroglutamic acid

Under normal conditions, OPA reacts with primary amides instantly, but not at all with imides. A prior treatment with sodium hypochlorite to open the ring structure is required to derivatize imides such as proline and hydroxyproline with OPA[8]. However, attempts to oxidize pyroglutamic acid with sodium hypochlorite prior to OPA derivatization were unsatisfactory; the OPA derivative, if it was formed at all, was barely fluorescent. After unsuccessful trials of other derivatization methods, such as the use of Dns chloride, we discovered that the most effective way to analyze pyroglutamic acid was to elute the underivatized pyroglutamic acid with 0.1% phosphoric acid and measure it with UV at 200 nm. Pyroglutamic acid was eluted in 15 min, practically free of interferences from other amino acids (Fig. 4). The detection response was linear in the concentration range of 100–500 pmol. Simultaneous analysis of all three amino acids (glutamine, glutamic acid and pyroglutamic acid) was not feasible, however, because both the strongly absorbing glutamine

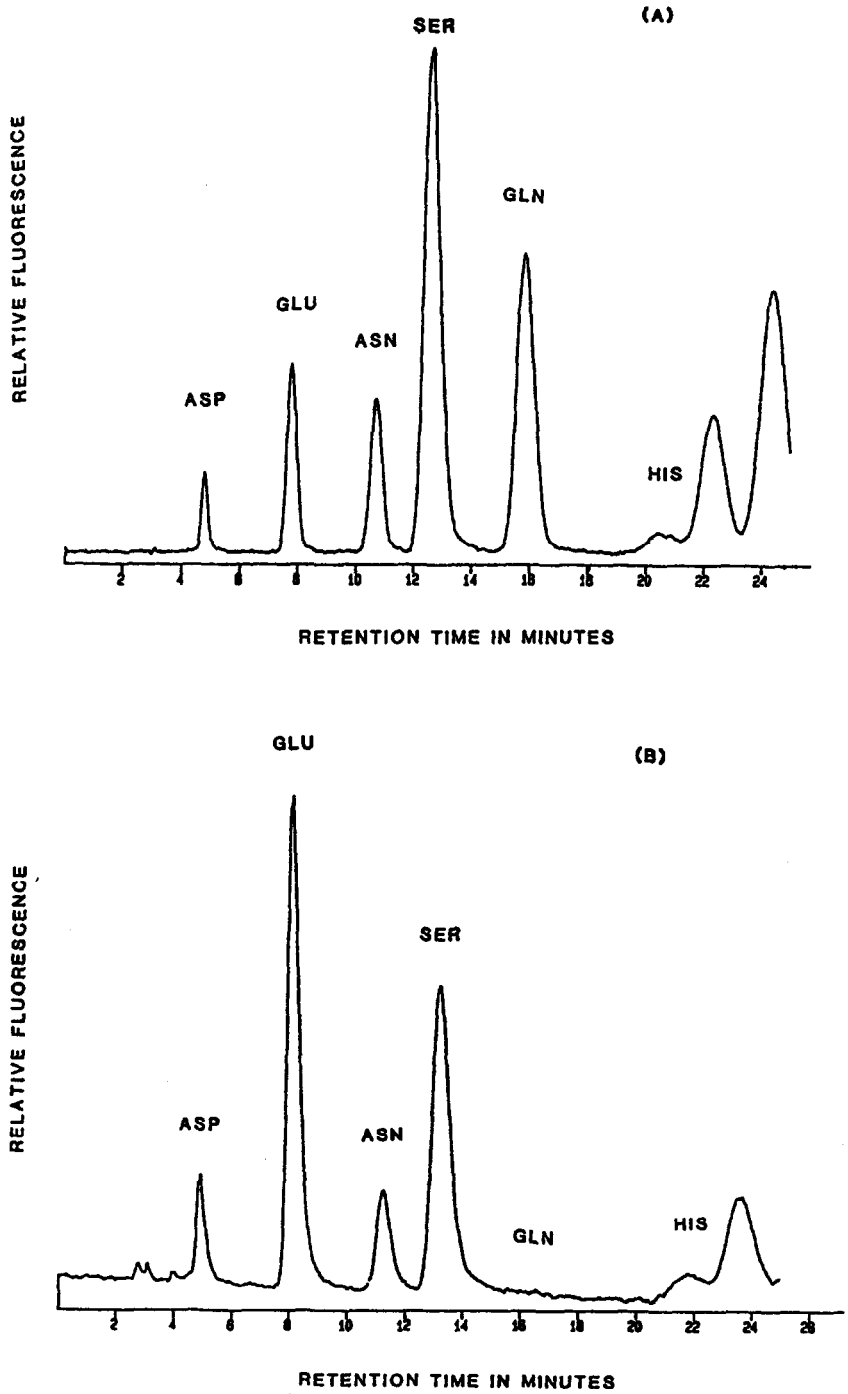

Fig. 3. Elution profiles of the OPA–ME derivatives of (A) casein hydrolyzed by pronase E and proteinase K, and (B) enzymatic casein hydrolysate from a commercial source. The mobile phase consisted of acetonitrile–0.04 *M* sodium acetate buffer, pH 5.40 (13.6:86.4).

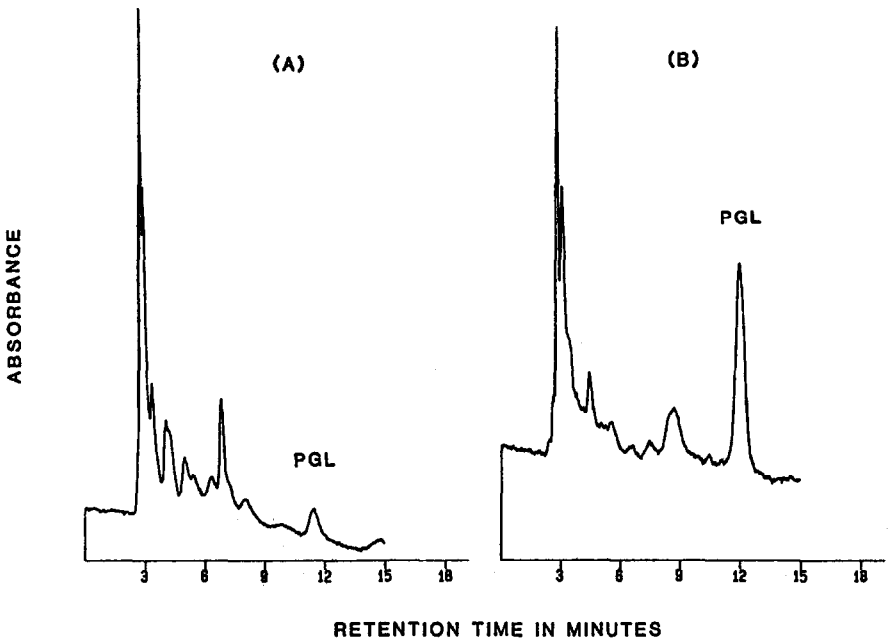

Fig. 4. Elution profiles of underivatized (A) casein hydrolyzed by pronase E and proteinase K, and (B) enzymatic casein hydrolysate from a commercial source. The mobile phase of the isocratic elution was 0.1% phosphoric acid. PGL = Pyroglutamic acid.

and the weakly absorbing glutamic acid, together with many other amino acids at various degrees of absorbance, were eluted almost unretained in about 3 min in a cluster of peaks.

The unaccountable gain in glutamic acid in the deamidated hydrolysate could be explained by the occurrence of pyroglutamic acid. Pyroglutamic acid could be present in the original protein or formed during or after the hydrolysis by the cyclization of glutamic acid and/or the deamidation–cyclization of glutamine. The presence of pyroglutamic acid in the casein hydrolysates was confirmed by HPLC analysis as shown in Fig. 4. In both the laboratory hydrolysate and the commercial hydrolysate, the amounts of pyroglutamic acid found fully accounted for the unaccountable gains in glutamic acid. Asparagine can be deamidated, though not as readily as glutamine, and there is no discrepancy in accounting for the asparagine and aspartic acid during deamidation because neither of these two amino acids are likely to form a cyclized product.

Stability studies

Experiments were conducted in which glutamine, glutamic acid and pyroglutamic acid were each dissolved in solutions of various pH values and the solutions stored at different temperature levels. The contents of the solution were analyzed periodically by methods described above to investigate the effects of pH and temperature on the interconversion among these three amino acids. The results showed that pyroglutamic acid was extremely stable; it could be converted to glutamic acid

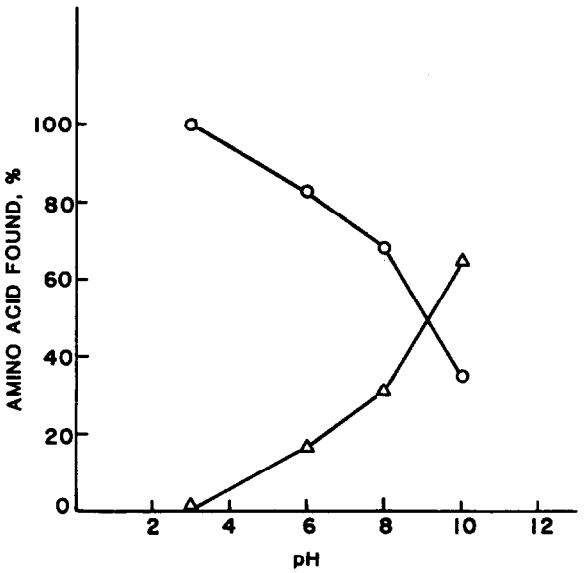

Fig. 5. Effect of pH on the recovery of glutamine (O) and the formation of pyroglutamic acid (△). The amino acids were determined after glutamine was stored in 0.1 *M* sodium phosphate buffer (adjusted to desired pH by phosphoric acid or sodium hydroxide) at 37°C for 24 h.

only under drastic conditions, such as in 2 *M* hydrochloric acid at 100°C for 2 h. Glutamic acid was stable at room temperature, but would cyclize to form pyroglutamic acid at elevated temperatures and prolonged storage. Glutamine was extremely unstable; it was deamidated readily and, at alkaline pH values, formed mostly the cyclized product of pyroglutamic acid.

Of particular interest is the formation of pyroglutamic acid under conditions of enzymatic protein hydrolysis. Fig. 5 shows what occurs to glutamine when stored in solutions of various pH values at 37°C for 24 h, the normal conditions for enzymatic hydrolysis. Glutamine loss increased with the increase of pH, and was almost totally accounted for by the appearance of pyroglutamic acid. If the reaction is conducted at pH 10.0, for instance, the reaction mixture could retain only 35% of its glutamine at the end of the reaction, with a 64% conversion to pyroglutamic acid and the remaining 1% to glutamic acid. Under similar conditions, glutamic acid was practically unchanged; only trace amounts of pyroglutamic acid could be detected at the pH range of 3 to 10. The conversions to pyroglutamic acid in a protein hydrolysis may not be the same as in experiments using free amino acids; however, indications are that substantial amounts of glutamine will be converted to pyroglutamic acid under conditions of high pH, long reaction time, and elevated temperature.

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REFERENCES

1 H. Wilson and R. K. Cannan, *J. Biol. Chem.*, 119 (1937) 309.
2 R. M. Achibald, *Chem. Rev.*, 37 (1945) 161.
3 T. Kasai and P. O. Larsen, *Acta Chem. Scand.*, B33 (1979) 213.
4 J. V. Benson, Jr., M. J. Cordon and J. A. Patterson, *Anal. Biochem.*, 18 (1967) 228.
5 M. H. Garner, W. H. Garner and F. R. N. Gurd, *J. Biol Chem.*, 248 (1973) 5451.
6 K. Nakamura, Y. Morikawa and I. Matsumoto, *Bunseki Kagaku (Jap. Anal.)*, 29(5) (1980) 314.
7 F. F. Shih and A. D. Kalmar, *J. Liquid Chromatogr.*, 7(6) (1984) 1169.
8 J. D. H. Cooper, M. T. Lewis and D. C. Turnell, *J. Chromatogr.*, 285 (1984) 484.