THE DETERMINATION OF PROLINE IN PROTEIN HYDROLYSATES WITH LACTOBACILLUS BREVIS

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Proline was first determined in 1901 by Fischer (2), who isolated copper L- and Dl-proline dihydrates from an ester fraction of an acid hydrolysate of casein. During the ensuing three decades proline was determined essentially by this procedure in the hydrolysates of more than 50 proteins by Fischer (1901-11), Abderhalden (1902-36), Osborne (1906-11), and other workers. That data of satisfactory analytical value were probably unobtainable by the ester method was demonstrated by Osborne and Jones (3), who were able to recover only about 73 per cent of the proline from a mixture containing eleven pure amino acids. Because of the difficulties encountered in the ester fractionation method, proline has been determined by isolation as the hydantoin (4), cadmium chloride salt (5), picrate (6), rhodanilate (7), and the mercury salt of its N-methyl derivative (8) from butanol extracts, copper salts, or protein hydrolysate fractions of other types. Dakin (9) determined proline by extracting protein hydrolysates with butanol and crystallizing the proline from ethanol solutions which had been treated with mercuric acetate and barium hydroxide to precipitate other monoamino acids. Jones and Johns (10) determined proline by calculation from the total and amino nitrogen values of hydrolysates which had been freed from the basic amino acids by precipitation with phosphotungstic acid. Guest (11) proposed a method based on the colorimetric determination of pyrrole obtained by oxidation of proline; however, proline and hydroxyproline could not be differentiated by this procedure.

The solubility product principle was adapted by Bergmann and Stein (12, 13) to the quantitative determination of proline in gelatin hydrolysates. Although the method appears to yield reliable data when the numerous precautions emphasized by these authors are observed, it has not found wide application. Extensive data on the recovery of N-acetylated proline from mixtures of acetylated amino acids have been reported by Synge et al. (14-16) and by Tristram (17). These authors stated that the proline values may be accurate to ±5 per cent. Polson et al. (18) have de-
**Table I**
Composition of Basal Medium*

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount per liter</th>
<th>Constituent</th>
<th>Amount per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>15.8 gm.</td>
<td>XH4PO4</td>
<td>0.3 mg.</td>
</tr>
<tr>
<td>L-Arginine HCl</td>
<td>920 mg.</td>
<td>MgSO4·7H2O</td>
<td>0.24 mg.</td>
</tr>
<tr>
<td>Natural asparagine H2O</td>
<td>520 mg.</td>
<td>FeSO4·7H2O</td>
<td>0.12 mg.</td>
</tr>
<tr>
<td>L-Cysteine HCl</td>
<td>1440 mg.</td>
<td>MnSO4·4H2O</td>
<td>0.006 mg.</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>600 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>1480 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Histidine HCl·H2O</td>
<td>60 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Isoleucine</td>
<td>260 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>260 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Lysine HCl</td>
<td>960 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>560 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>2680 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Norvaline</td>
<td>360 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Phenylalanine</td>
<td>400 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Serine</td>
<td>560 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Threonine</td>
<td>500 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Tryptophan</td>
<td>1380 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>160 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Valine</td>
<td>480 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>1880 mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>52.5 gm.</td>
<td>AGTU mixture§</td>
<td>120 mg.</td>
</tr>
<tr>
<td>d-Glucose, anhydrous</td>
<td>45.0 gm.</td>
<td>Adenine</td>
<td>30 mg.</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>7.5 gm.</td>
<td>Guanine</td>
<td>30 mg.</td>
</tr>
<tr>
<td>Phosphate buffer salts¶</td>
<td>45.0 gm.</td>
<td>Thymine</td>
<td>30 mg.</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.6 gm.</td>
<td>Uracil</td>
<td>30 mg.</td>
</tr>
<tr>
<td></td>
<td>0.3 gm.</td>
<td>IX mixture∥</td>
<td>60 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoxanthine</td>
<td>30 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthine</td>
<td>30 mg.</td>
</tr>
</tbody>
</table>

*All the constituents were of C.P. quality. The medium is prepared conveniently as follows from the indicated quantities of constituents. Suspend the amino acids in 600 ml of distilled water, add 10.0 ml of the adenine-guanine-thymine-uracil solution, and stir the mixture until the amino acids dissolve. Add the glucose and sodium acetate and stir the suspension until the solids dissolve. Add 3.0 ml of phosphate buffer salts solution, 3.0 ml of mineral salts solution, 10.0 ml of hypoxanthine-xanthine solution, 1.00 ml of the vitamin solution, and the arabinose. Stir the mixture until the solids dissolve, adjust the pH of the solution to 6.8, and dilute the solution to 1 liter with distilled water. Steam, but do not autoclave, the final solution for 20 minutes at 100°. The concentration of the constituents of the basal medium in the final 3.0 ml volumes of assay solutions was two-thirds that indicated in Table I.

† 0.1 n HCl solution containing 4.0 gm. of MgSO4·7H2O, 0.200 gm. of FeSO4·7H2O, and 0.200 gm. of MnSO4·4H2O per 100 ml.
TABLE I—Concluded

<table>
<thead>
<tr>
<th>Solution Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>† 50 per cent ethanol solution containing 45.0 mg. of calcium dl-pantothenate,</td>
</tr>
<tr>
<td>450 mg. of nicotinamide, 1.00 mg. of folic acid, 0.075 mg. each of biotin and p-amino-</td>
</tr>
<tr>
<td>benzoic acid, and 6.0 mg. each of the other vitamins listed per liter.</td>
</tr>
<tr>
<td>§ 0.5 N HCl solution containing 0.30 gm. each of adenine, guanine, thymine, and</td>
</tr>
<tr>
<td>uracil.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>¶ Distilled water solution containing 10.0 gm. of K$_2$HPO$_4$ and 10.0 gm. of KI$_2$PO$_4$ per 100 ml.</td>
</tr>
</tbody>
</table>

scribed a method for the determination of proline by paper chromatography and ninhydrin analysis.

With the advent in 1943 of microbiological methods for the determination of amino acids it seemed a reasonable expectation that a satisfactory procedure could be developed for proline. That *Leuconostoc mesenteroides* P-60 might be employed for this purpose was indicated in 1944 by the report (19) that proline was essential for the growth of this organism. Two other lactic acid bacteria, *Lactobacillus arabinosus* 17-5 (20) and *Lactobacillus fermenti* 36 (21), have been shown not to require proline. Values have been reported, subsequently, for proline,$^1$ determined microbiologically with *Leuconostoc mesenteroides* P-60 and different basal media, including one described by the present authors (19). While the data obtained for proline in corn steep liquor (25), *botulinum* toxin (26), urine of mice (27) and humans (28), plant viruses (29), and purified proteins (30–34) appear to be satisfactory for comparative purposes, there is little basis, other than approximate agreement with literature values, on which to judge their probable accuracy.

Because of the observation (35) in 1947 that proline was synthesized by *Leuconostoc mesenteroides* P-60 after prolonged incubation on an enriched basal medium, it seemed probable that this amino acid could not be determined with high precision and accuracy with this organism and any available near optimal or suboptimal basal medium. It was conceivable that the incompleteness of the latter might be overcome by nutrients introduced into the assay solution by the test sample. In searching for a more promising organism a systematic study$^2$ was made of the nutritional requirements of five organisms which, as had been shown previously, synthesized proline only slowly even on an enriched basal medium (35) and produced a relatively large amount of acid on the same medium containing arabinose rather than glucose (36). The organisms investigated

$^1$ Proline has been determined in several proteins by Brand et al. (22), who employed a prolineless mutant of *Neurospora crassa* and an unpublished method. A mutant strain of *Escherichia coli* which grows only in the presence of proline has been investigated by Tatum (23) and by Simmonds and Fruton (24).

$^2$ To be reported in a forthcoming paper.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Per cent proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein I</td>
<td>10.3 (3.1)</td>
</tr>
<tr>
<td>Casein II</td>
<td>10.7 (2.2)</td>
</tr>
<tr>
<td>Silk Fibroin I</td>
<td>0.55 (1.0)</td>
</tr>
<tr>
<td>Silk Fibroin II</td>
<td>0.58 (1.2)</td>
</tr>
<tr>
<td>Casein I hydrolyzed** + 4% proline</td>
<td>101 (2.3)</td>
</tr>
<tr>
<td>Casein II hydrolyzed** + 4% proline</td>
<td>98 (2.8)</td>
</tr>
<tr>
<td>Silk Fibroin II hydrolyzed** + 4% proline</td>
<td>96 (4.9)</td>
</tr>
<tr>
<td>Casein test mixture††</td>
<td>95 (2.7)</td>
</tr>
<tr>
<td>Casein test mixture †† × 4</td>
<td>93 (2.1)</td>
</tr>
<tr>
<td>Casein test mixture †† × 5</td>
<td>101 (1.5)</td>
</tr>
<tr>
<td>Casein test mixture †† × 10</td>
<td>96 (1.6)</td>
</tr>
<tr>
<td>Casein test mixture †† × 15</td>
<td>96 (1.2)</td>
</tr>
<tr>
<td>Silk fibroin test mixture††</td>
<td>99 (1.6)</td>
</tr>
</tbody>
</table>

*The purified sample of L-proline employed as the standard contained negligible moisture and ash. Its nitrogen content was 99.6 per cent of the theoretical amount and the specific rotation was [α]_25° = −85.23° in water where c = 1.4951, l = 4.0, and α = −5.097°. Another sample of L-proline, obtained through the courtesy of Dr. E. E. Howe (Merck and Company), was reported to be 99.5 per cent pure according to phase rule solubility determination. The specific rotation of this sample was found to be [α]_25° = −84.79° and its activity towards Lactobacillus brevis 97.1 per cent that of the authors' preparation. Each value recorded for Casein I, Silk Fibroin I, casein test mixture × 4, and casein test mixture × 10 is a single determination. Each value recorded for Casein II, Silk Fibroin II, casein test mixture × 5, casein test mixture × 15, and silk fibroin test mixture is the average of two closely agreeing percentages found in separate experiments. Each value recorded for casein test mixture × 1, casein test mixture hydrolyzed plus proline, and casein test mixture plus proline hydrolyzed is the average of three closely agreeing percentages found in separate experiments. Each figure in the parentheses represents the mean deviation from the mean found for proline at the different levels of sample.

† n-Proline (6 to 34 γ) in n-proline, hydroxy-L-proline (10 to 5000 γ), n-ornithine HCl (10 to 5000 γ), and L-pyroglutamic acid (8 to 4000 γ) were inactive within detectable limits towards Lactobacillus brevis.

‡ Described previously (38), containing 6.21 per cent moisture, 0.55 per cent ash, and 15.60 per cent nitrogen corrected for moisture and ash.

§ Prepared essentially as described previously (38) and containing 9.66 per cent moisture, 0.63 per cent ash, and 15.35 per cent nitrogen corrected for moisture and ash.

|| Described previously (38), containing 5.68 per cent moisture, 0.25 per cent ash, and 18.7 per cent nitrogen corrected for moisture and ash.
TABLE II—Concluded

** By heating approximately 1.0 gm. sample of protein or amino acid test mixture with 10 ml. of 5.97 N HCl for 18 hours at 120° in a Leiboff urea tube in an electrically heated Leiboff urea apparatus.

†† Composition essentially the same as that given previously (38).

††† Composition essentially the same as that given previously (39), except for the addition of 2.8 per cent of L-aspartic acid, 0.40 per cent of L-tryptophan, and 6.8 per cent of DL-valine.

were Lactobacillus lycopersici (4005),* Lactobacillus pentoaceticus (367), Leuconostoc mesenteroides P-60 (8042), Leuconostoc citrovorum (8081), and Lactobacillus brevis (8257). A microbiological procedure for the determination of proline with Lactobacillus brevis and the arabinose-containing basal medium given in Table I is described in the present paper.

EXPERIMENTAL

The assay techniques were essentially the same as those described by Dunn et al. (37). The protein hydrolysates, the inoculum suspensions and solutions of the basal media, amino acid test mixtures, the standard amino acid and sodium chloride were delivered to 4 inch test-tubes with the aid of a Brewer automatic pipette (Baltimore Biological Laboratories). All solutions, except the basal media, were adjusted to the same concentration of sodium chloride to compensate for any stimulatory or inhibitory effects of this salt. The standard (fifteen levels), the amino acid test mixtures (ten levels), and the protein hydrolysates (ten levels) were each run in quadruplicate or triplicate.

All solutions (final volume, 3.0 ml. per 4 inch test-tube) were adjusted to pH 6.8 and sterilized by being steamed for 20 minutes at 100°. Solutions autoclaved for 10 minutes were colored deep orange. The inoculum was prepared by inoculating the previously described medium (37) with growth from a tomato juice-agar stab, incubating the inoculated solution for 24 hours at 35°, centrifuging the suspension, and washing the cells once with saline. Each assay tube was inoculated with 0.10 ml. of a suspension of the washed cells diluted with saline to an optical density equivalent to about 90 per cent transmission. The inoculated tubes were incubated for 72 hours at approximately 34°.

Satisfactory assay values were obtained over the range, 6 to 42 γ, of proline. At the 42 γ level of proline, acid was produced equivalent approximately to 19 ml. of 0.03 N NaOH (per 3.0 ml. final volume of solu-

* American Type Culture Collection number.
Acid equal to about 1.7 ml. of 0.03 N NaOH was formed in each blank tube. Acid production in the standard and test sample tubes differed by $1.5 \pm 0.9$ per cent. The experimental results are shown in Table II.

**DISCUSSION**

The described assay procedure is considered to be satisfactory because acid production was relatively high, the standard curves were smooth and uniform except for a lag period up to 3 days of proline, relatively little acid was formed in the blank tubes, and there was no evidence of any stimulatory or inhibitory effects. The recoveries of proline from amino acid test mixtures simulating the composition of casein and silk fibroin averaged 97 (93 to 102) per cent and the recovery of proline added to two different casein and two different silk fibroin samples, before and after hydrolysis of the protein or the mixtures, averaged 100 (96 to 104) per cent.

Some additional observations of particular significance were that the activity of L-proline was inappreciably altered by heating it for 18 hours at 120° with 6 N HCl in the presence and absence of other amino acids, and that hydroxy-L-proline, D,L-ornithine, and L-pyroglutamic acid were inactive towards *Lactobacillus brevis*. The authors' method was found adaptable without significant decrease in precision or probable accuracy to assays of a protein (silk fibroin) and amino acid test mixtures with relatively low (about 0.5 per cent) proline content.

### Proline in Casein

The proline found in casein (corrected for moisture and ash) averaged 10.5 (10.3 and 10.7) per cent for two samples of casein. This value is in good agreement with that (10.6 per cent) which Tristram found by partition chromatography for a sample of casein containing 15.65 per cent nitrogen. This percentage of proline was considered to be reliable to $\pm 2$ per cent. Other values for proline in casein, both higher and lower than the percentage obtained in the present experiments, have been reported. These percentages include (a) 11.6 ± 0.7 (32), 11.2 (34), and 9.36 (31), determined microbiologically with *Leuconostoc mesenteroides* P-60, (b) 8.2 (11) and 8.1 (41) determined by colorimetric analysis of a degradation product (pyrrole), (c) 4.7 to about 10 (42-47), determined by calculation from the non-amino nitrogen of an ester fraction soluble in ethanol, (d) 6.7 (8) determined as the mercury double salt of stachydrin (dimethyl-betaine of proline), and (e) 0.6 to 3.5 (1, 48-50) determined by isolation of the copper salt from an ester fraction.

—Quoted by Chibnall (40).
Proline in Silk Fibroin

The proline found in silk fibroin (corrected for moisture and ash) averaged 0.57 (0.55 and 0.58) per cent for two samples of silk fibroin. The proline content of silk fibroin prepared from silk proteins obtained from different sources has been found to vary from about 0.7 to 2.5 per cent by Abderhalden et al. (50–60) and other investigators (61, 62) on the basis of the copper salt isolated from ester fractions.

SUMMARY

A microbiological method has been described for the determination of proline in protein hydrolysates with Lactobacillus brevis. It was found that the proline content of moisture- and ash-free casein and silk fibroin was 10.5 and 0.57 per cent, respectively.

BIBLIOGRAPHY

1. Rockland, L. B., and Dunn, M. S., Food Tech., in press.
CORRECTIONS

In the articles beginning on pages 1, 23, 33, and 43, Vol. 168, No. 1, April, 1947, and in the article beginning on page 11, Vol. 179, No. 1, May, 1949, read *Lactobacillus brevis* (8287) for *Lactobacillus brevis* (8267).

In Vol. 180, No. 3, October, 1949, on page 1154, legend to Fig. 6, read *Sample V* for *Sample VI*; on page 1155, legend to Fig. 7, read *Sample VI* for *Sample VII*; on page 1156, second paragraph, read *fasting 18 hours and 4 hours* for *fasting 4 hours and 18 hours*.

In Vol. 181, No. 1, November, 1949, on page 282, third structure,

\[
\begin{align*}
\text{read} & \quad \begin{array}{c}
\text{OH} \\
\text{CH}_2\text{COOH}
\end{array} \\
\text{for} & \quad \begin{array}{c}
\text{OH} \\
\text{CH}_3\text{COOH}
\end{array}
\end{align*}
\]
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