J. Nutr. Sci. Vitaminol., 19, 251–262, 1973

LYSINE-LACTOSE BROWNING PRODUCTS
I. PROPERTIES OF THE REACTION PRODUCTS

Teruko FUJIWARA-ARASAKI1 and C. B. COULSON2

Kobe Yamate Women’s College, Kobe, Japan and Arthur D. Little Research Institute, Inveresk, Midlothian, Scotland, U. K.

(Received December 2, 1972)

Fairly typical Maillard-type reaction products were obtained from the lysine-lactose browning system. These substances gave positive ninhydrin (17), orcinol (16) and LOWRY (19) reactions and fraction D1, P1 and P3 showed fairly higher sugar content. Fractions D, and P1 contain 22.4 and 21.8% of sugar, respectively. Fractions D1 and P1 yielded 6.95 and 9.44% of free lysine, respectively, i.e. this corresponded to 25 and 24% of their original total nitrogen levels. A preliminary in vitro test of the fractions (P1 and P3) using M. tuberculosis H37Pv suggests some growth stimulation properties.

Quite number of studies on the amino-carbonyl reaction between the amino group of amino acids and the glycosidic center of sugars have been reported during the last fifty years. Reviews of these studies covering various aspects, including commercial and biological implications, have been published (1–3).

Recently, activity against micro-organisms has been found to be present in some of these reaction products (4, 9, 22, 23). The products resulting from heating glycine-dextrose inhibit the growth of Phytophthora fragaria (4), and heated skim-milk inhibits the growth of L. bulgaricus-09 (5), while N-D-glucosyl-glycine stimulates the growth of L. gayoni (6). It was recently demonstrated that the product of lysine-xylose browning system has stronger antimicrobial activity for Staphylococcus aureus 209 p (23). Reviews on the Bifidus factors (7, 8) indicate that milk-whey has nitrogen-containing oligosaccharides which act as a growth factor for certain strains Lactobacillus bifidus and that “Papain hydrolysed casein” also contains similar factors. HABERLAND (9) pointed out that N-(4-carboxy-3-hydroxyphenyl)D-glucosyl-amine has an antitubercular activity. These findings suggest that the amino-carbonyl or the Maillard reaction produce either inhibitors or stimulators of micro-organisms growth.

We have used the lysine-lactose browning system as a possible guide to the

1 新崎輝子
2 National College of Food Technology, University of Reading, Weybrige, Surrey, U. K.
improvement in purification of anti-mycobacterial factors in milk-whey (10, 11). The present paper reports on the properties of melanoidin-like substances resulting from the browning reaction in lysine-lactose systems and their possible biological activity in relation to Mycobacterium. Preliminary reports of this work have been published by Fujiwara and Coulson (10, 11).

EXPERIMENTAL AND RESULTS

Materials

Lysine and lactose. A standard analytical grade of lysine·HCl and lactose (both British Drug Houses Ltd.) were used.

Ion-exchange materials. Dowex-50 × 2 and × 8, 50–100 mesh; Amberlite IRC-50 × E 64, (100–200 mesh); DEAE-Sephadex A-50, medium (Pharmacia, Uppsala); were used.

Methods

Preparation of dried Dowex–50 × 2 resin. Dried resin was prepared according to the method of Dixon (12). Dowex–50 × 2 was recycled four times between 4 N NaCl and 4 N HCl, left in acid overnight at 27°C, washed with distilled water until the washings were free of Cl⁻, dried with acetone, and stored at 0°C.

Paper chromatography. Whatman No. 1 and Toyo-Roshi No. 51–A papers were used for one-dimensional paper chromatography. The solvents used were:

S₁ = 1-butanol-acetic acid-water (4: 1: 5, v/v/v upper phase)
S₂ = 1-butanol-pyridine-water (4: 6: 3, v/v/v)

The detection was carried out using aniline hydrogen phthalate for sugars (13) and 0.2% ninhydrin in acetone for amino acids.

Absorption spectra. Absorption spectra were determined using the Hilgar manual and Shimadzu (Beckman type) UV Spectrophotometers, and the Hitachi Infra-red Spectrometer.

Estimation of lysine content. Amberlite IRC 50 column chromatography was used for the estimation of lysine in fraction P₁ (ISHII (14)) and, Dowex–50 × 8 column chromatography (1.0 × 15 cm column, 0.1 M phosphate buffer, pH 6.8) was used for that in fraction D₁ (MOORE and STEIN (15)).

Test of activity on microorganisms. The tests for activity on microorganisms were kindly carried out by Dr. J. D. Sleigh of the Bacteriology Department, University of Edinburgh, Scotland, using the Disc method (26). Discs 5 mm in diameter used for test were cut from Whatman No. 1 filter paper (such discs were able to absorb about 0.01 ml of fluid). The amounts of fractions per disc are given as follows:
All solutions were sterilised prior to use with the aid of a Hemmings centrifugal filter (Beaumaris Instrument Company, Beaumaris, Anglesey) fitted with "Oxoid" cellulose acetate discs.

1. Preparation of lysine-lactose browning reaction products
   
a) Fractionation on paper chromatography. Citrate buffer (0.5 M, pH 4.3; 8 ml) was added to a mixture of lysine HCl (266.7 mg) and lactose (754.8 mg) and the resultant solution was heated in a sealed tube (120-130°C; 1.5 hr).

   The brownish coloured reaction mixture was subjected to paper chromatography (solvent, S1 for 72 hr, descending). Six brownish bands were detected on

\[
\begin{array}{|c|c|c|}
\hline
\text{Concentration of test solutions(mg/ml)} & \text{Amount per disc (µg)} \\
\text{0.01 M phosphate buffer, pH 7.4)} & \\
\hline
P_1 & 2.8 & 28 \\
P_3 & 10.8 & 100 \\
\hline
\end{array}
\]

Fig. 1. Paper chromatogram of slow-moving brownish compound obtained from lysine-lactose reaction mixture. (ninhydrin in acetone spray). Solvent; 1-butanol-acetic acid-water (4: 1: 5, v/v/v), upper layer, descending, 72 hr.
the chromatogram as shown in Fig. 1. When the paper chromatogram was treated with ninhydrin reagent, bands 1, 2, and 3 showed weak reactivity towards ninhydrin, while band 4 and the band between 4 and 5 gave strong colours with

Fig. 2. Absorption spectra of fraction D1, P1, and P3 obtained from lysine-lactose reaction mixture. A: fraction P1 B: fraction P3 C: fraction D1

Fig. 3. Elution diagram for chromatography of lactose-lysine reaction product on DEAE-Sephadex A-50. (Column 2 × 50 cm). 0.1 M Pyridine-acetate buffer (pH 5.0) was used. ------at 420 m\(\mu\), --- ninhydrin at 570 m\(\mu\), --- orcinol at 540 m\(\mu\).
ninhydrin. This band between bands 4 and 5 was coincident with authentic lysine. This was probably unreacted lysine. Bands-1 (designated P1) and 3 (designated P3) were cut out and eluted from the paper with water and the eluates evaporated to dryness in vacuo. P1 had a deep brownish colour, while P3 was light brown. Nitrogen content of P1 and P3 were found 6.48 and 4.59%, respectively. Their absorption spectra are given in Fig. 2. The absorption maxima of P1 (A in Fig. 2) and P3 (B in Fig. 2) occur at 320 m\textmu and 350 m\textmu in both cases. The 320 m\textmu peak of P1 is higher than that of P3, while P3 possesses a higher 350 m\textmu peak.

b) Fractionation by DEAE Sephadex A–50 column chromatography. The sample was prepared by the same browning system mentioned in (a). 874 mg of lysine·HCl, 1570 mg of lactose and 8 ml of 0.5 M citrate buffer (pH 4.3) were used in this experiment.

DEAE-Sephadex A–50 used was regenerated with 0.5 N HCl and then 0.5 N NaOH, followed by a water wash, then treated with 0.1 M pyridine acetate buffer (pH 5.0) and finally packed into a column (2.5 × 50 cm). The reaction product was adjusted to pH 5 with 1 N NaOH, placed on the column and the elution carries out with the same buffer. The effluent was collected in 5 ml fractions, and

![Paper chromatogram of the fraction obtained by DEAE-Sephadex column chromatography. Solvent used 1-butanol-acetic acid-water (4:1:5, v/v/v; upper layer, descending, 48 hr). Detection reagents; 0.2% ninhydrin in acetone, aniline hydrogen phthalate.](image-url)
0.3 ml portions of these fractions were examined by orcinol (16) and ninhydrin tests (17). The brown colour was estimated at 420 m\(\mu\) by spectrophotometrically. The elution diagram is shown in Fig. 3.

The three fractions obtained from the product by chromatography on a DEAE-Sephadex A–50 column, were examined by one-dimensional paper chromatography using solvent, \(S_1\) (48 hr, descending). Paper chromatogram of these fractions is given in Fig. 4.

Fractions I, II and III are designated to \(D_1\), \(D_2\) and \(D_3\), respectively. Fraction \(D_1\) gave only one spot at the origin which was positive to both ninhydrin and aniline hydrogen phthalate spraying reagents (13). Fraction \(D_2\) was obtained nine ninhydrin positive and six aniline hydrogen phthalate positive spots, whilst fraction \(D_3\) gave eight and five, respectively. The absorption spectrum of \(D_1\) is given in Fig. 2. The absorption maxima of \(D_1\) occur at 290 m\(\mu\) and 320 m\(\mu\) i.e. shorter than that of \(P_1\) and \(P_2\). The elemental analysis of \(D_1\) is given in Table 1. This \(D_1\) value has some similarity to other Maillard reaction products (18).

### Table 1. Elemental analysis of the fraction \(D_1\) and Maillard reaction products.

| C (\%) | H (\%) | N (\%) | O (\%) | Reference          |
|--------|--------|--------|--------|-------------------|
| 56.28  | 5.84   | 5.33   | 32.55  | Authors           |
| 58.6   | 4.6    | 4.4    | 32.4   | Maillard (18)     |
| 59.1   | 5.0    | 5.5    | 30.4   |                   |
| 58.9   | 5.1    | 6.0    | 30.4   |                   |

### Table 2. Analytical data on certain fractions in the lysine-lactose browning system.

| Test                      | \(D_1\) | \(P_1\) | \(P_3\) |
|---------------------------|---------|---------|---------|
| orcinol (16) (\%)         | 22.4    | 21.8    | 35.6    |
| (after resin hydrolysis)  | 13.5    | 8.9     |         |
| Ninhydrin (17) (\%)       | 6.8     | 11.4    |         |
| (after 5.7 N HCl, 6 hr    | 1.8     | 2.8     |         |
| hydrolysis)               |         |         |         |
| LOWRY (19) (\%)           | 2.370   | 3.730   | 0.850   |

*a* glucose values.

*b* leucine values.

*c* The values indicate the optical density at 500 m\(\mu\) per mg of samples.

2. **Analytical values of \(D_1\), \(P_1\) and \(P_3\)**

Fractions, \(D_1\), \(P_1\) and \(P_3\) were examined by means of the orcinol (16), ninhydrin (17) and LOWRY (19) tests and the analytical values are summarized in Table 2. Fractions \(D_1\), \(P_1\) and \(P_3\) were found to contain 22.4, 21.8 and 35.6\% of sugar, respectively (calculated as a glucose) by the orcinol method, and \(D_1\) and \(P_1\) 6.8 and 11.4\% of amino acid (calculated as a leucine) by the ninhydrin method, while
D₁, P₁ and P₃ had ratios of 2.370, 3.730 and 0.850 of the optical density (at 500 m\(\mu\)) per mg of materials by the Lowry method, respectively. Fractions D₁ and P₁ (5 mg in 2.0 ml water) were added to 200 mg of dry Dowex-50 × 2 resin and then hydrolysed in sealed tubes (100°C for 1 hr). The resulted solutions were examined by means of orcinol and ninhydrin tests. Both D₁ and P₁ released 13.5 and 8.9% of sugar, respectively, with only trace of ninhydrin positive material. Both fractions were found to contain only galactose (paper chromatography using solvent S₂) as shown in Fig. 5. Galactose released from both D₁ and P₁ corresponded to 60 and 40% of the original sugars respectively. However, hydrolysing the original material with 5.7 N HCl at 100°C for 6 hr, both D₁ and P₁ were almost completely destroyed, and this resulted in lower values for orcinol (3.3 and 2.5%) and ninhydrin (1.8 and 2.8%) tests.

![Fig. 5. Paper chromatogram of sugars released by resin (Dowex-50 × 2) from D₁ and P₁. (Solvent: 1-butanol-pyridine-water, 6: 4: 3, v/v/v, for 20 hr, detection reagent: aniline hydrogen phthalate).](image)

3. **Lysine content in fractions D₁ and P₁**

Lysines in fractions D₁ and P₁ were examined by means of column chromatography (ISHII (14) and MOORE-STEIN method (15) respectively). Lysines fractions from D₁ and P₁ were prepared as follows: Both fractions were first heated with dry Dowex-50 × 2 resin in water (sealed tube, 100°C, 1 hr) then washed with water. Then the basic components were eluted with 2 N NH₄OH and the eluates evaporated in vacuo. The residues were hydrolysed with 5.7 N HCl (105–110°C for 6 and 19 hr, respectively). The elution patterns of basic
Table 3. Lysine and total nitrogen contents of the fractions, D₁ and P₁

|          | D₁  | P₁  |
|----------|-----|-----|
| Total-N (%) | 5.33| 6.48|
| Lysine (%)  | 6.95| 9.44|
| Lysine-N (%)| 1.35| 1.85|

fractions are shown in Figs. 6 and 7. Lysine values are summarized in Table 3. Fractions P₁ and D₁ were found to contain 9.44 and 6.95% of lysine, respectively, in addition to some neutral amino acids-like material. Lysine-N in P₁ and D₁ was found to be 1.85 and 1.35%, respectively i.e. 24 and 25% of total-N, respectively. Hydrolysing D₁ with dry resin (in water, 100°C, 1 hr), resulted in a 3.3% value for lysine, when this was followed by complete hydrolysis with 5.7 N HCl (105–110°C for 6 hr), 6.95% of lysine was found in addition to the increased neutral amino acid fractions as shown in Fig. 7.

4. **Infrared absorption spectra**

The infrared absorption spectra (KBr disk) of P₁ and D₁ are shown in Fig. 8. Both samples showed characteristic absorption bands representing –OH (3300–3400, 2940 cm⁻¹), C–O and C–O–C (1000–1150 cm⁻¹) and pyranose ring (875,775 cm⁻¹) of glycosidic compounds. Amide (3300–3400, 1640 cm⁻¹) and carboxyl groups (1720, 1400 cm⁻¹) were detected in both samples.
5. Biological activity vis-a-vis microorganisms

The effect of substances P₁ and P₃, which resulted from the lysine-lactose browning reaction, were examined on the following microorganisms *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus faecalis, Escherichia coli, Proteus mirabilis, Pseudomonas pyocyanea* and *Mycobacterium tuberculosis, H₃7Rv*, using the disc method. After overnight incubation at 37°C of *S. aureus, S. pyo-

![Graph showing elution pattern for chromatography of D₁ on Amberlite IRC 50. Column: 1 x 30 cm, Solvent: citrate-phosphate buffer (a:b, 1.5:1). a: 0.2 M citrate buffer, pH 6.0. b: 0.2 M phosphate buffer, pH 6.0. Hydrolysis was carried out with 5.7 N HCl at 105-110°C for 6 hr.](image-url)
genes, *S. faecalis, E. coli, P. mirabilis* and *P. pyocyanea* there were no zones of growth inhibition. The test for the virulent *Mycobacterium tuberculosis, H₃₇Rv* was seeded in Kirchner's liquid culture medium (27) to which was added 10% serum albumin. The inoculum was a 1 in 100 dilution of a 10-day culture of the *H₃₇Rv* strain of *M. tuberculosis* in fluid Dubos medium (27). Growth of small rough buff colonies on both P₁ and P₃ was first observed after only 4 days incubation. These colonies were actually, growing on the surface of the disc and when filmed they were shown to be rather large and fat acid- and alcohol-fast bacilli. Subsequently growth took place all over the slope but there was marked enhancement in the area surrounding the disc. This experiment was repeated and although growth enhancement was again observed it was not nearly so marked-colonies first became visible after some 10 days on this occasion.

**DISCUSSION**

The products D₁ and P₁ from lysine-lactose browning reaction were slow-moving on paper chromatograms developed with 1-butanol-acetic acid-water (4: 1: 5, upper phase, for 72 hr), gave fairly typical Maillard-type reaction products (18) (initial stage) on elemental analysis (Table 1), and were water soluble and acidic in character (pH 3).

The absorption maxima of P₁ and P₃ occur at 320 and 350 mµ in both cases, but D₁ weak absorption maxima at 290 mµ and 320 mµ. Previously, AMBLER (20) reported the absorption maximum of D-xylose-glycine, a Maillard product, as 320 mµ and those of the D-glucose-glycine system as having 400 mµ. The absorption maxima at 290 mµ and 320 mµ could be based on that of furfuraldehyde resulting from lactose degradation. BOWNES (21) has reported that hexoses, pentoses and uronic acids, when heated with sulfuric acid, yield 5-hydroxy-methylfurfuraldehyde, 5-furfuraldehyde and 5-formylfuroic acid which possess absorption maxima at 295–8 mµ, 317 mµ and 321–3 mµ, respectively. CHUYEN et al. (24) reported recently that a product, 2-(2'-oxo-pyrazin-1'-yl) isocaproic
caproic acid which shows an absorption maximum at 322 m\(\mu\) was produced through amino-carbonyl reaction between dipeptide and glyoxal. KATO (25) also pointed out that the substituted pyrrole-2-aldehyde occurred in amino-carbonyl reaction. It is suggested that these substances may occur in the present lysine-lactose system.

Sugar contents of the three fractions D\(_1\), P\(_1\) and P\(_3\) were fairly high (22.4, 21.8 and 35.6\% respectively). Values for galactose released by mild acid-hydrolysis using Dowex-50 \(\times\) 2 were found to be 13.5\% and 8.9\% in D\(_1\) and P\(_1\), respectively. These values for galactose correspond to 60\% and 40\% of original sugar values of D\(_1\) and P\(_1\).

On the other hand, values of 6.95 and 9.44\% of lysine were obtained for D\(_1\) and P\(_1\) respectively, as a result of complete acid hydrolysis. Lysine-N constituted 24 and 25\% of the total-N of P\(_1\) and D\(_1\) (Table 3). After complete acid-hydrolysis, the neutral amino acid fractions increased were found on column chromatography (ISHII (14) and MOORE-STEIN (15) method (Fig. 7)).

Mild hydrolysis of D\(_1\) with Dowex-50 \(\times\) 2 resin at 100\°C for 1 hr released less than 3.30\% of lysine. It is interesting to note that the lysine residue seemed to be linked firmly the core of the melanoid substances.

What is also interesting is that, on preliminary testing with virulent Mycobacterium tuberculosis, H37Rv both P\(_1\) and P\(_3\)-enhanced growth.

This work was supported in part by a grant from the Wellcome Trust, London. The authors express sincere thanks to the director of Arthur D. Little Research Institute, Dr. F.N. Woodard, C.B.E., F.R.S.E. for his interest in and support of this work, Dr. J. D. Sleigh of the Bacteriology Department, University of Edinburgh, Scotland for the test of micro-organisms, and Professor Y. Matsushima, Faculty of Science, Osaka University, Japan for the elemental analysis, and Infra-red spectrophotometry.

REFERENCES

1) ELLIS, G. P., Advances in Carbohydrate Chem., 14, 64 (1959).
2) ELLIS, G. P. and HONEYNIAN, J., Advances in Carbohydrate Chem., 10, 95 (1955).
3) HODGE, J. E., Agr. Food Chem., 1, 928 (1953).
4) McKeeN, W. E., Science, 123, 509 (1956).
5) Viswanathan, L. and Sarma, P. S., Nature, 180, 1371 (1957).
6) Roger, D., King, T. E., and Chieldelin, V. H., Proc. Soc. Exptl. Biol. Med., 82, 140 (1953).
7) Bell, D. J., Annu. Rep. Progr. Chem., 52, 333 (1955).
8) Clamp, J. R., Hough, L., Hickson, J. L., and Whistler, R. L., Advances in Carbohydrate Chem., 16, 159 (1961).
9) Haberland, G., Arzneimittel-Forsch., 1, 298 (1951).
10) Fujibara, T. and Coulson, C. B., Biochem. J., 88, 61p (1963).
11) Fujibara, T. and Coulson, C. B., Biochem. J., 88, 62p (1963).
12) Dixon, A. S. J., Biochem. J., 60, 165 (1955).
13) Partridge, S. M., Nature, 164, 443 (1949).
14) Ishii, S., J. Biochem. (Tokyo), 43, 531 (1956).
15) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951).
16) Sørensen, M. and Hauggard, G., *Biochem. Z.*, **260**, 247 (1933).
17) Yemm, E. W. and Cocking, E. C., *Analyst*, **80**, 209 (1955).
18) Mailard, L. C., *Ann. Chim.* (Paris), **5**, 258 (1916).
19) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randoll, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
20) Ambler, J. A., *Ind. Eng. Chem.*, **21**, 47 (1929).
21) Bowness, J. M., *Biochem. J.*, **70**, 107 (1958).
22) Haneoka, Y., *J. Ferment. Technol.* (Japan), **46**, 909 (1968).
23) Kuwabara, S., Simizu, U., and Yajima, M., *J. Agr. Chem. Soc. Japan* (in Japanese), **46**, 89 (1972).
24) Chuyen, N. V., Kurata, T., and Fujimaki, M., *Agr. Biol. Chem.* (Japan), **36**, 1257 (1972).
25) Kato, H., *Agr. Biol. Chem.* (Japan), **31**, 1086 (1967).
26) Baker, F. J., Handbook of Bacteriological Technique, Butterworths, London, p. 186 (1969).
27) Baker, F. J., Handbook of Bacteriological Technique, Butterworths, London, p. 91-92 (1967).