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Identification of Nutritional Components in Trypticase Responsible for Recovery of *Escherichia coli* Injured by Freezing[1]

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ABSTRACT

Moss, C. WAYNE (North Carolina State University, Raleigh), AND M. L. SPECK. Identification of nutritional components in Trypticase responsible for recovery of *Escherichia coli* injured by freezing. J. Bacteriol. **91**:1098–1104. 1966.—Freezing and storage of *Escherichia coli* at −20 C resulted in nonlethal or "metabolic" injury to a proportion of the surviving population. The injury was manifested as an increased nutritional requirement after freezing. Injured cells could not grow on a minimal agar medium, but could develop on Trypticase Soy Agar. The percentage of injured survivors varied among strains, but was little affected by altering the freezing menstruum. Trypticase was found to be the component in Trypticase Soy Agar responsible for the recovery of injured cells, and contained five closely related peptides that possessed most of the biological activity. Isolation of the peptides was accomplished by Sephadex gel chromatography, paper chromatography, and high-voltage paper electrophoresis. Hydrolysis of the peptides destroyed the ability to restore injured cells.

Straka and Stokes (19) noted that destruction of bacteria by exposure to subzero temperatures proceeded through intermediate stages of nonlethal injury to eventual death of the cell. Injured survivors were described as "metabolically" injured, since such cells became more demanding in their nutritional needs. Similar phenomena resulting from freezing have been observed by other workers (1, 13, 14, 17) with several bacterial species and with the use of appropriate minimal and complex media to determine injured cells. In each of these studies, greater numbers of cells were recovered on the complex media, indicating that these contained substances which contributed to the recovery of injured cells.

The purpose of this study was to identify those factors in Trypticase Soy Agar that were responsible for recovery of strains of *Escherichia coli* injured by freezing. The effects of several variables (prior growth medium, freezing menstruum, time and temperature of freezing and storage) on injury and death are also reported.

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MATERIALS AND METHODS

Organisms. E. coli 451-B was obtained from Robert P. Straka of the U.S. Department of Agriculture Western Regional Research Laboratory, Albany, Calif. Strains A, Y, and X of *E. coli* were freshly isolated from raw milk.

Growth and plating media. Cultures were grown in either Trypticase Soy Broth (TSB) or in a minimal medium broth (MB). The individual ingredients of the two media were purchased in bulk, allowing for a uniform composition throughout all experiments. TSB (*p*H 7.1) contained 1.5% Trypticase (BBL), 0.5% Phytone (BBL), and 0.5% NaCl (J. T. Baker Chemical Co., Phillipsburg, N.J.). MB (*p*H 7) contained: K_2HPO_4 (Fisher Scientific Co., Fair Lawn, N.J.), 7.0 g; KH_2PO_4 (Fisher), 3.0 g; sodium citrate (Baker), 0.1 g; $MgSO_4 \cdot 7H_2O$ (Baker), 0.1 g; $(NH_4)_2SO_4$ (Baker), 1.0 g; and glucose (Baker), 2.0 g. The glucose was autoclaved and added separately. Trypticase Soy Agar (TSA) and minimal medium (MA) were used as plating media and had the same composition as the TSB and MB, respectively, with the exception of the addition of 1.5% agar (Difco).

Freezing menstrua. The freezing menstrua were selected from those in which freezing injury has been reported previously. These were: distilled water (13),

0.85% NaCl (17), 0.5% beef extract (19), and 0.038 M potassium phosphate buffer (20). The *p*H of the phosphate buffer was 7.6 with an ionic strength of 0.1.

Freezing and storage. Volumes of 5 ml of the cell suspensions were frozen and stored in screw-capped tubes (20 by 120 mm) at −20 C in a freezer for various intervals extending up to 28 days. The cooling rate was approximately 1.4 C per min to 0 C, and 0.5 C per min from 0 to −20 C.

Determination of injury and death. Injury and death were determined by colony counts on TSA and MA after 48 hr of incubation at 35 C. The unfrozen cultures produced the same number of colonies on both agar media, but after freezing and storage fewer colonies could be recovered on the MA. The various states of the bacterial cells after exposure to freezing temperature were defined according to Straka and Stokes (19): (i) unharmed cells were those which grew on MA after freezing; (ii) the number of dead cells was the difference between the plate count on TSA before and after freezing; (iii) the number of injured cells was determined by the difference in plate count on TSA and MA. This differential in counts on the two media after freezing and subsequent incubation for 48 hr at 35 C remained unchanged by longer incubation periods ranging up to 7 days at 22, 35, 37, or 40 C.

Determination of biologically active components in Trypticase. Components active in the recovery of injured cells were determined by an adaptation of the bioautographic method of Kennedy, Speck, and Aurand (6). This method was used to locate areas of biological activity on paper chromatograms and on paper sheets from high-voltage electrophoresis. Frozen-and-thawed cells were diluted and added to 200 ml of MA to give a final concentration of 8 to 10 injured cells per milliliter. The cells and agar were mixed and poured into a sterile Pyrex baking dish (22 by 34 cm) with a glass cover. Paper strips from chromatography or electrophoresis were placed on the surface of the solidified agar for 10 to 30 min, then removed, and the plates were incubated at 35 C. After 48 hr, the plates were observed for biologically active zones which were detected by an increase in the number of colonies which developed around these areas.

Isolation of active components from Trypticase. The active components in Trypticase responsible for the recovery of injured cells were isolated by use of Sephadex G-25 gel and paper chromatography, and high-voltage paper electrophoresis. The fractions were examined for carbohydrates with anthrone reagent (12). The presence of peptides was detected with the biuret test, or by heating chromatograms for 30 to 50 min at 70 C after staining with ninhydrin (0.25% in acetone). Free amino acids were also detected with ninhydrin.

Sephadex (120 g) was treated with 0.05 M NaCl (2) and the resulting fines were repeatedly removed until the supernatant fraction was clear. The washed Sephadex was packed into a column (4 by 60 cm) and equilibrated for 24 hr with the eluent (0.1 N NH_4OH) before the sample was applied. A 10-ml amount of sterile 10% Trypticase was applied to the bed surface of the column, and 8 ml of eluate was collected per tube at a flow rate of 1.5 ml/min. The absorption pattern of the column eluate was continuously recorded at 265 mμ. Tubes were combined into fractions according to the absorption spectra, and each fraction was flash-evaporated at 45 C to 3 ml. Each fraction was filtered through a Seitz or Millipore filter and tested for ability to recover injured cells by additon to MA.

The active fraction from the Sephadex column was spotted as a compact band on Whatman no. 3 MM chromatography strips (3.8 by 60 cm), and then developed for 16 to 18 hr at room temperature by descending chromatography with the upper phase of the butanol-acetic acid-water (5:1:4) solvent. After drying, a marker strip was cut from the edge of each chromatogram and stained with ninhydrin, and the ninhydrin-positive areas were marked on the unstained portion of each chromatogram. Biologically active areas were located on the unstained portion of random chromatograms by bioautography. Areas on the chromatograms where activity was present were removed and eluted with distilled water. The eluate was concentrated under reduced pressure and rechromatographed on paper as described above. The developed chromatograms were dried, and marker strips were removed to locate ninhydrin-positive and biologically active zones. The latter areas were removed from the remaining chromatograms, eluted with distilled water, concentrated to 5 ml, Seitz-filtered, and checked for ability to recover injured cells by addition to MA.

High-voltage electrophoresis. The active fraction from the second run on paper chromatography was applied as a band across the entire width of a sheet (46 by 57 cm) of Whatman no. 3 MM paper. Pyridine-acetic acid-water (1:10:289) buffer (*p*H 3.6), described by Katz, Dreyer, and Anfinsen (5), was applied to the paper evenly up to the band, so that the sample was covered simultaneously from both sides. The paper was then placed in the tank of a high-voltage electrophoresis apparatus (High Voltage Electrophorator, model D, Gilson Medical Electronics, Middleton, Wis.) containing the buffer covered with Varsol. Prior to and during operation, the tank was maintained at 20 to 24 C by circulating a coolant (3 C) through coils contained in the Varsol. A potential of 3,000 v and a current of 150 to 160 ma was maintained for 3 hr. The paper was removed and dried, and a 2-cm lengthwise strip was cut off from each side and stained with ninhydrin. A 5-cm lengthwise strip was also cut off and tested for active areas by bioautograph. The active areas on the remainder of the sheet were removed and eluted. The eluates were lyophilized and were then placed in a vacuum desiccator over Drierite and P_2O_5.

Amino acid identification. Positive identification of the amino acids in hydrolysates of the active peptides obtained by electrophoresis was made with the use of an amino acid analyzer (model 120 B, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.). The number of residues in the original peptides was estimated by the procedure described by Gordon and Speck (13).

Assay procedure. Each strain was grown into the

stationary phase (15 hr) in TSB or MB at 35 C. The cells were centrifuged under refrigeration, washed three times with 0.85% NaCl, and resuspended in the desired freezing menstruum. Samples (5 ml) containing cells and suspending fluid were frozen (−20 C), thawed for 5 min at 24 C after various intervals of storage, and plated in triplicate on each agar medium. Plates were incubated for 48 hr at 35 C, and all visible colonies were counted. Frozen-and-thawed cells for bioautograph were obtained by the same procedure.

RESULTS

Extent of injury during storage. The numbers of survivors that showed injury after freezing and storage varied among the strains tested (Table 1). The relative number of injured survivors generally increased through 2 days of storage, but remained somewhat constant over the remainder of the storage period. Growth media and freezing menstrua were not measurably influential on the percentage of cells that showed injury after 7 days of storage. The percentage of injured survivors was greatest for strain Y, and remained relatively constant during storage. Therefore, strain Y was selected as the assay strain in the isolation of the factors in TSA which allowed injured cells to grow.

Determination of active components in TSA. Since all strains gave higher colony counts on TSA after freezing, Trypticase and phytone were added to MA to test for their ability to permit recovery of injured cells. Vitamin-free acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) enriched with tryptophan was also added to MA to determine whether amino acids permitted growth of injured bacteria. The results (Table 2) showed that Trypticase enabled the MA medium to recover as many cells as TSA. Similar results were obtained on injured cells in the other strains of *E. coli*. Phytone and acid-hydrolyzed casein were inactive for injured bacteria.

Isolation of active component(s) from Trypticase. The initial step in the isolation procedure was the filtration of Trypticase through Sephadex, by which three fractions absorbing at 265 mμ were obtained (Fig. 1). Each fraction contained biuret-positive material, but the color test was more intense with fractions 1 (100 to 180 ml) and 2 (180 to 300 ml) than with fraction 3 (300 to 450 ml). Each fraction gave a color reaction with ninhydrin which increased in intensity from fractions 1 through 3. Carbohydrate was not detected in any fraction. Each fraction was passed through a Seitz filter and lyophilized. On the basis of dry weight, 35% of the original material added to the column was in fraction 1, 32% in fraction 2, and 33% in fraction 3, with no loss occurring by the filtration. Based on these values, each fraction was reconstituted in sterile distilled water and added to MA in the same concentration as was present in TSA. Observations were then made on the ability of each fraction to promote growth of injured cells in MA. The results (Table 3) indicated that fraction 2 contained much more activity for recovery of *E. coli* Y than either of the other two fractions. Addition of the three fractions to MA gave recovery comparable to that obtained with TSA.

Paper chromatograms of fraction 2 were prepared and developed with butanol-acetic acid-water (5:1:4) and were tested on MA bioautograph plates to locate the area(s) which was active for the recovery of injured cells. The results

TABLE 1. *Influence of the growth medium, freezing menstruum, and time of frozen storage on nonlethal injury of Escherichia coli*

Strain	Growth medium	Freezing menstruum	No. of trials	Days of storge (−20 C)				
				1	2	3	7	14
451-B	TSB	0.85% NaCl	5	32*	53	45	40	45
	TSB	0.5% beef extract	7	16	31	46	48	49
	TSB	0.038 M phosphate buffer	2	20	29	—	30	38
	TSB	Distilled water	4	9	23	25	28	35
	MB	Distilled water	4	37	36	38	43	40
Y	TSB	0.5% beef extract	8	75	80	70	75	64
	TSB	0.038 M phosphate buffer	6	55	70	65	62	70
	MB	0.5% beef extract	5	79	84	77	77	82
X	TSB	0.5% beef extract	3	32	36	—	36	32
	TSB	Distilled water	1	9	10	24	23	40
A	TSB	0.5% beef extract	3	25	55	50	48	60

* Percentage of survivors injured.

TABLE 2. *Effect of plating medium on recovery of Escherichia coli Y after freezing and storage (−20 C) in 0.5% beef extract*

Plating medium	Unfrozen control	Days of storage (−20 C)				
		1	2	3	4	7
TSA	130	39*	23	24	10	9
MA	130	14	4	6	3	3
MA + 2% Trypticase	130	39	21	23	9	9
MA + 0.5% Phytone	130	14	3	7	3	4
MA + 2% acid-hydrolyzed casein	140	13	5	8	3	4

* Number of organisms per milliliter ($\times 10^5$). Each figure represents the average of triplicate trials, with each trial being the average of three plates.

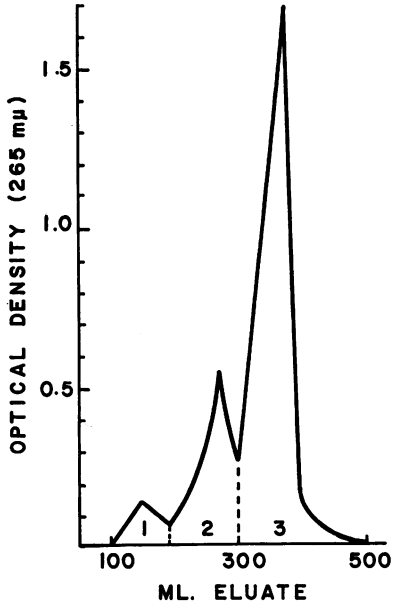

FIG. 1. *Elution pattern of Trypticase through a Sephadex G-25 column with 0.1 N ammonium hydroxide as eluting agent.*

from the use of several chromatograms showed only one consistent, yet definite, area of recovery, which extended from R_F 0.36 to R_F 0.62. Several ninhydrin-positive bands of varying color intensity were located in this active area, but no band was distinctly separated. Concentrated eluate of this area from chromatograms gave a strong positive biuret test.

Eight ninhydrin-positive zones were detected when the active fraction (R_F 0.36 to 0.62) was rechromatographed with the butanol-acetic acid-water solvent. The zones at R_F 0.41 (zone 5), R_F 0.50 (zone 6), R_F 0.58 (zone 7), and R_F 0.67 (zone 8) were active for the recovery of injured cells when tested by bioautography, and each gave a positive biuret test. Zone 5 was much more active than the other three zones, and stained only lightly with ninhydrin at room temperature. Zones 7 and 8 also stained lightly with ninhydrin, whereas zone 6 gave a very strong reaction. There was an overlapping of zones 5 and 6, which suggested that the lower part of zone 5 (designated zone 6) was similar to the upper portion of the zone, and that the strong reaction was perhaps due to the presence of a free amino acid. Acid hydrolysis and subsequent two-dimensional paper chromatography of each zone showed a higher concentration of tyrosine in zone 6, which would account for the strong reaction with ninhydrin. The material present in each of the active zones was eluted, concentrated, Seitz-filtered, and tested for activity to promote growth of injured cells by addition to MA. The results (Table 4) confirmed previous results with bioautography, in that zone 5 gave substantially more recovery (83%) than the other zones. The activity of zone 6 was possibly due to overlapping with zone 5. Zones 7 and 8 also contained active material which appeared to be a peptide, as suggested by a strong biuret test and weak reaction with ninhydrin.

Further separation of zone 5 by paper chromatography was unsuccessful with butanol-acetic acid-water or with butanol-2-butanone-water (2:2:1) as described by Mizell and Simpson (11). In both solvents, the zone moved as one band; however, the band was not sharp and exhibited some trailing, indicating that the material was not homogeneous.

Material in zone 5 was separated into seven distinct and well-defined ninhydrin-positive bands

TABLE 3. *Recovery of cold-injured Escherichia coli Y by eluates from fractions of Trypticase separated by filtration through Sephadex G-25*

Plating medium	Unfrozen	Days of storage (−20 C)		
		1	3	10
TSA	320	150*	9.1	3.2
MA	310	20	2.3	0.8
MA + fraction 1†		80	4.1	0.9
MA + fraction 2		140	8.0	2.9
MA + fraction 3		90	4.5	1.4
MA + fractions 1, 2, 3		160	8.8	3.1

* Number of organisms per milliliter ($\times 10^5$).

† The amount of each fraction added was 15 mg per ml of medium.

by high-voltage paper electrophoresis. Each of the bands moved toward the cathode, and each gave a positive biuret test, indicating that the separated components were peptides. Five of the seven peptides were active when tested by bioautograph, and were designated E1, E2, E3, E4, and E5. Material corresponding to each of these active peptides was eluted from 20 large electrophoresis sheets, concentrated, and lyophilized, and a small portion was hydrolyzed separately with acid and base; the remainder was used for quantitative amino acid analysis. Subsequent two-dimensional chromatography of the alkaline hydrolysates indicated that tryptophan was not present in either of the peptides.

Amino acid composition of peptides. The amino acid composition of the five active peptides was determined initially by conventional two-dimensional paper chromatography, and subsequently with the use of an amino acid analyzer. The probable numbers of constituent amino acids in the peptides are presented in Table 5. Proline, glutamic acid, and valine generally were present in the highest concentration in each peptide except E1, which contained only a trace of proline. Tyrosine and phenylalanine were present only in trace amounts in each peptide except in E5, which contained approximately two residues of phenylalanine. The quantities of alanine and serine in each peptide generally were the same, but the quantity of the other amino acids varied from one peptide to the other. Assuming the number of residues to be correct and rounding off the value to the nearest whole residue, the approximate molecular weight of each peptide was as follows: E1, 1,900; E2, 3,000; E3, 2,800; E4, 2,600; and E5, 3,100. These approximate values are probably too low, in that traces of tyrosine and phenylalanine also were present. The similarity of the molecular weights would help to explain the movement of the peptides as one band on paper chromatography. The hydrolyzed peptides were inactive in the recovery of injured cells when tested by addition to MA.

TABLE 4. *Recovery of Escherichia coli Y by eluates* from active zones on paper chromatograms*

Plating medium	Colony count × 10^7/ml†	Injured cells recovered
		%
TSA	78	
MA	30	(62) ‡
MA + zone 5	70	83
MA + zone 6	61	65
MA + zone 7	38	17
MA + zone 8	44	29

* The amount of eluate added per plate was 0.2 ml.

† Each figure represents the average of five trials, with each trial being the average of duplicate plates.

‡ Represents percentage of survivors injured. None was recovered.

DISCUSSION

The results of this study show that the phenomenon of metabolic or nonlethal freezing injury noted previously (1, 13, 14, 17, 19) may occur generally with strains of *E. coli* under certain experimental conditions. Each strain used in our study showed a high percentage of injured survivors after 14 days of storage at −20 C, but

TABLE 5. *Amino acid composition of acid hydrolysates of active peptides as determined by the amino acid analyzer*

Amino acid	Peptide				
	E1	E2	E3	E4	E5
Proline	Trace*	5.6	7.0	5.1	5.3
Glutamic acid	3.0	3.1	5.3	4.9	2.5
Valine	2.0	1.9	2.8	2.1	5.8
Alanine	1.1	1.0	1.9	1.4	2.0
Serine	2.1	1.6	1.0	1.3	1.0
Aspartic acid	3.1	4.3	1.0	1.2	1.0
Isoleucine	1.1	3.9	1.3	1.1	1.1
Threonine	1.0	Trace	1.0	1.0	Trace
Glycine	1.0	2.4	1.0	1.0	3.9
Leucine	1.5	1.0	1.0	3.3	1.2
Tyrosine	Trace	Trace	Trace	Trace	Trace
Phenylalanine	Trace	Trace	Trace	Trace	1.8

* Residues for each amino acid, calculated by dividing the micromole values obtained for each amino acid in each separate peptide by the lowest micromole value for the amino acid present in that peptide.

the extent of injury was dependent upon the strain. Marked differences in survival among strains of *Streptococcus lactis* exposed to subzero temperature also have been noted (13). Such strain differences may have contributed to the marked differences among the results of various studies on effects of low temperature upon bacteria (4, 21).

In certain respects, the present results differ from those of Straka and Stokes (19) and Arpai (1), who noted a progressive decrease in the number of injured cells during storage at −18 C. However, our results showed an increase in the percentage of injured survivors after 1 day of storage, and this remained relatively constant through 14 days. The data of Nakamura and Dawson (14) for freezing injury of *Shigella sonnei* suspended in skim milk showed a gradual decline, during storage, in the number of cells recovered on synthetic medium, whereas the number recovered on complex media remained constant after approximately 5 days. The differential in colony counts on the two media (injured cells), therefore, increased during storage, which is consistent with our findings. However, in our experiments and those of Nakamura and Dawson (14), cells were frozen very slowly (−20 C) and thawed slowly (24 C), whereas Straka and Stokes (19) froze the cells more rapidly (−78 C). This difference in the rate of freezing, which is known to be an important factor affecting the survival of other microorganisms (9), could account for the conflicting results.

Our results indicate that Trypticase was the component in TSA responsible for the recovery of injured cells, and that it contained five closely related peptides that possessed most of the activity. The close similarity of the active peptides was revealed by the appearance of one band on paper chromatograms with two different solvents, and by the approximate molecular weights from amino acid analyses. Even though each peptide gave a distinctly separated band by electrophoresis, an assessment of complete homogeneity of the peptide(s) is limited by the trace quantities of the amino acids proline, threonine, phenylalanine, and tyrosine obtained from the amino acid analysis. However, it is possible that these amino acids were not present in all molecules of the peptide(s), which would account for trace amounts relative to the other amino acids. Phillips and Gibbs (16), working with peptides from trypsinized casein, noted that the aromatic amino acids constituted only a small proportion of the total peptide material. Even though the peptides isolated in the present study were responsible for most of the recovery, other active material(s) was also present in Trypticase. This material(s) also appeared to be peptide or a peptide-like compound, but this does not preclude the possibility that other compounds such as amino acids may also contribute to the activity of Trypticase. However, this appears to be unlikely in view of negative results obtained with acid-hydrolyzed casein, and the loss in activity when the active peptides were hydrolyzed.

The function of peptides in the recovery of cells injured by freezing is not clear. Biologically active peptides have been observed in numerous studies, but their mode of action in most instances remains obscure (7, 10, 15). The biological activity of peptides has been considered a result of their involvement in an active transport mechanism (8) and their protection from bacterial amino acid deaminases and decarboxylases (18). In a recent study, Postgate and Hunter (17) noted that the lag period for frozen-and-thawed cells was greatly increased over that of unfrozen cells and that death occurred during this lag time on both rich and minimal media. However, death during an extended lag phase was greater on the minimal medium, suggesting that the rich medium contained factors which prevented death during lag. Perhaps the peptides manifest their activity by stimulating cell repair and growth during the lag phase, thereby preventing death which would occur in their absence. This is supported by the work of Arpai (1), who observed that the extended duration of the lag phase for frozen-and-thawed cells was proportional to the number of metabolically injured cells.

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