Exopeptidase Profiles of Bifidobacteria

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(Received June 17, 1985)

Summary The exopeptidase activities of five different strains of bifidobacteria occurring habitually in healthy human intestinal canal were measured on 61 synthetic substrates. The cluster analysis, based on the results, indicates that four strains, with the exception of Bifidobacterium adolescentis a M101-4, have similar exopeptidase profiles. All CFE from these five strains contained at least three kinds of aminopeptidases (aminopeptidase with broad substrate specificity, aminopeptidase hydrolyzing selectively X-Pro type and aminopeptidase hydrolyzing selectively Pro-X type) and carboxypeptidase.

Key Words exopeptidase, aminopeptidase, carboxypeptidase, bifidobacteria, intestinal flora, cluster analysis

Bifidobacteria are the predominant bacteria occurring habitually in the intestinal canal of man and various other animals. Various studies have been made as to physiological implication, influence on human health and clinical application of bifidobacteria (1). The proteolytic systems of bifidobacteria play an important role in the digestion of proteins and peptides in the intestinal canal of the host. However, few studies have been made on the exopeptidases in bifidobacteria.

The present paper describes the profiles of exopeptidases in CFE obtained from five strains of different species of bifidobacteria of human origin.

MATERIALS AND METHODS

Chemicals. The following substrates were used: Gly-Phe, Leu-Gly-Gly, Gly-2-NA (Protein Research Foundation, Osaka), Ala-Ala-Ala-His, Ala-Gly-Gly-Gly (Bachem Fine Chemical, Inc.), Ala-, Leu-4-NA (Merck Japan Ltd., Tokyo), cbz, carbobenzyloxy; Disc-PAGE, polyacrylamide-gel electrophoresis.

Abbreviations: CFE, cell-free extract; 2-NA, 2-naphthylamide; 4-NA, 4-nitroanilide; cbz, carbobenzyloxy; Disc-PAGE, polyacrylamide-gel electrophoresis.
Gly-Pro (Tokyo Kasei, Tokyo), Ala-Ala, Ala-Leu, Ala-Phe, Arg-Asp, Glu-Ala, Gly-Ser, Glu-Val, His-Ala, His-Leu, His-Ser, Leu-Leu, Leu-Ala, Gly-Ser, Glu-Val, His-Ala, Hist-Leu, His-Ser, Leu-Leu, Leu-Phe, Leu-Val, Lys-Gly, Lys-Leu, Phe-Ala, Tyr-Ala, Tyr-Glu, Tyr-Leu, Val-Ala, Val-Gly, Val-Leu, DL-Ala-DL-Leu-Gly, Glu-Gly-Phe, Glu-Val-Phe, Gly-Gly-Val, Gly-Phe-Ala, Leu-Leu-Leu, Phe-Val-Val, Phe-Pro, Val-Pro, Pro-Ala, Pro-Gly, Pro-Leu, Pro-Tyr, Pro-Val, Lys-4-NA, Ala-, Arg-, Glu-, Leu-, Phe-, Pro-2-NA, bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), cbz-Gly-Gly, cbz-Gly-Phe, cbz-Gly-Val (Sigma Chem. Co.). L-Amino acids were used with the exception of glycine when the type of amino acid was not specified. All other reagents were of guaranteed grade.

Organisms. Bifidobacterium adolescentis a M101-4, B. bifidum a A234-4, B. breve a 1-53-8, B. infantis a 1-10-5, and B. longum a M101-2 were supplied by the Japan Bifidus Foundation, Tokyo.

Preparation of CFE. The organisms were incubated in a stock medium (GAM broth, Nissui-Seiyaku, Tokyo) at 37°C under anaerobic conditions. After three successive subcultures, the organisms were cultured in GAM broth at 37°C for 35 h (stationary phase).

The cells were harvested by centrifugation at 4,000 × g for 30 min at 4°C, and washed three times with sterile saline (0.85% NaCl). The washed cells suspended in 0.05 M sodium phosphate buffer at pH 6.0 were lyophilized and stored in a desiccator at 4°C until used.

The dried cells were resuspended in 0.05 M sodium phosphate buffer at pH 6.0 (0.5% suspension w/v) and sonicated for 30 min in a Toyoriko sonifier, Tokyo (type N-50-6, 100 V, 10 kV). The temperature did not exceed 10°C. The disrupted cell preparation was centrifuged at 35,000 × g for 20 min at 4°C. After centrifugation the supernatant (CFE) was decanted and dialyzed overnight against the same buffer.

Assay of enzyme activities. The enzyme activity was measured by the colorimetric method of Goldberg and Rutenburg (2) for aminoacyl-2-naphthylamides, and by the spectrophotometric method of Foissy (3) for aminoacyl-4-nitroanilides. The activity of enzyme on various peptides with the exception of dipeptides containing the proline residue was measured by the ninhydrin method of Matheson and Tattrie (4). The activity of enzyme on the dipeptides containing proline residue was measured by the method of Yaron and Mlyner (5). The activity was measured at 30°C.

A unit (U) of activity was defined as the amount of enzyme which produces either 1 µmol 2-naphthylamine, 1 µmol nitroaniline, 1 µmol proline, or ninhydrin-positive substance corresponding to 1 µmol leucine, depending on the substrates used. In Disc-PAGE, the activity was expressed as the amount of released material (µmol)/min/ml extracted liquid.

Assay of protein. Protein concentrations were measured by the method of Lowry et al. (6) using bovine serum albumin as the standard.

Cluster analysis. Cluster analysis was carried out using the HITAC-M-200H
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computer at the Computer Center, the University of Tokyo, with the biomedical computer program package BMDP 1M (HITAC).

Disc-PAGE. Disc-PAGE was carried out according to the procedure of Davis (7) with 7.5% gel at pH 8.0. The protein (200 μg/gel) was added on the top of the gel and constant current was applied at 2 mA/gel for 2 h in a cold room (4°C). The gel was cut into 2 mm slices followed by enzyme extraction from the sliced gel with 0.01 M sodium phosphate buffer of pH 6.0 (1 ml/sliced gel).

RESULTS

Substrate specificity

Table 1 shows the activities of the exopeptidases in CFE obtained from five strains of different species of bifidobacteria against 61 kinds of synthetic substrates. Similar substrate specificity was found for all of these five strains with different levels of activity. High hydrolyzing activities were observed against dipeptides containing Leu, Phe, Tyr or Val in the N-terminal, while the activities against substrates containing Arg or Gly therein were low. Hydrolytic activity was also observed against dipeptide containing proline. The activity was especially high against the peptides containing proline in the N-terminal. Tripeptides and tetrapeptides were hydrolyzed rapidly. However, peptides containing Gly resisted hydrolysis by these enzymes. Hydrolytic activity was also observed against aminoacyl derivatives and bradykinin, and to a lesser extent against cbz-X-X type peptides.

Cluster analysis

Inter-species cluster analysis was made on the base of the results shown in Table 1. Figure 1 shows the results of cluster analysis expressed by a dendrogram. The amalgamation distance among these five strains was nearest for B. infantis a 1-10-5 and B. longum a M101-2 followed by B. breve a 1-53-8, B. bifidum a A234-4 and B. adolescentis a M101-4 in that order.

B. adolescentis a M101-4 had a slightly different aminopeptidase profile from

![Fig. 1. Dendrogram of Bifidobacterium by analysis of exopeptidase activities on synthetic substrates. Calculation was by computer program "BMDP 1M." Vol. 31, No. 6, 1985]
Table 1. Activities of cell-free extract from *Bifidobacterium* strains for various synthetic substrates.

|        | Ala-Ala | Ala-Leu | Ala-Phe | Arg-Asp | Glu-Ala | Glu-Val | Gly-Phe | Gly-Ser | His-Ala | His-Leu | His-Ser |
|--------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| *B. adolescentis* | 26.27   | 22.09   | 16.27   | ND      | 20.82   | 27.94   | 7.04    | 3.42    | 49.17   | 46.73   | 76.33   |
| *B. bifidum*       | 79.79   | 59.66   | 58.89   | ND      | 50.35   | 78.24   | 10.45   | 1.83    | 39.77   | 49.21   | 57.71   |
| *B. breve*         | 35.46   | 52.13   | 35.83   | 1.95    | 18.34   | 38.15   | 6.38    | ND      | 14.31   | 30.26   | 19.52   |
| *B. infantis*      | 27.41   | 56.85   | 33.67   | 1.42    | 38.02   | 32.53   | 2.72    | ND      | 11.79   | 35.66   | 24.28   |
| *B. longum*        | 31.15   | 61.12   | 40.99   | 1.99    | 47.17   | 43.07   | ND      | ND      | 22.16   | 39.41   | 38.96   |

|        | Leu-Leu | Leu-Phe | Leu-Val | Lys-Gly | Lys-Leu | Phe-Ala | Tyr-Ala | Tyr-Glu | Tyr-Leu | Val-Ala | Val-Gly |
|--------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| *B. adolescentis* | 42.54   | 30.87   | 41.28   | ND      | 19.60   | 50.91   | 75.07   | 59.21   | 20.41   | 32.94   | 52.58   |
| *B. bifidum*       | 175.84  | 123.55  | 156.49  | 3.66    | 5.29    | 182.02  | 206.83  | 134.00  | 84.83   | 163.07  | 209.15  |
| *B. breve*         | 161.20  | 73.65   | 107.48  | 3.21    | 20.70   | 97.52   | 86.78   | 67.63   | 65.31   | 93.57   | 108.66  |
| *B. infantis*      | 178.73  | 82.31   | 116.02  | 1.59    | 19.56   | 106.63  | 82.31   | 71.33   | 83.08   | 104.64  | 121.51  |
| *B. longum*        | 199.75  | 82.88   | 136.19  | 5.77    | 25.82   | 139.04  | 94.71   | 83.65   | 99.84   | 142.70  | 154.21  |

|        | Val-Leu | DL-Ala-DL-Leu-Gly | Glu-Gly-Phe Val-Gly-Phe | Gly-Gly-Val | Gly-Gly-Phe Ala-Leu-Gly | Leu-Leu-Leu Phe-Gly-Gly Val-Gly-Gly Val-Tyr-Val |
|--------|---------|--------------------|-------------------------|-------------|--------------------------|-----------------------------------------------|
| *B. adolescentis* | 27.08   | 1.83               | 6.34                    | 3.86        | 1.42                     | 6.67                                         | 4.27                                         | 40.46                                         | 9.60                                        | ND                                           | 6.26                                         |
| *B. bifidum*       | 105.37  | 19.72              | ND                      | 7.16        | 0.41                     | 2.72                                         | 5.61                                         | 297.07                                       | 3.46                                        | 1.63                                        | 81.33                                       |
| *B. breve*         | 99.88   | 23.87              | ND                      | 10.25       | ND                       | 11.14                                        | 5.37                                         | 261.89                                       | 5.98                                        | 0.81                                        | 64.09                                       |
| *B. infantis*      | 114.48  | 20.78              | 0.81                    | 7.04        | 0.41                     | 12.53                                        | 8.50                                         | 274.78                                       | 7.44                                        | 0.81                                        | 57.22                                       |
| *B. longum*        | 136.19  | 26.68              | ND                      | 10.49       | 0.41                     | 19.28                                        | 13.26                                        | 314.19                                       | 10.65                                       | ND                                           | 56.57                                       |
Bradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; 2-NA, 2-naphthylamide; 4-NA, 4-nitroanilide; cbz, carbobenzoxy; ND, not detected. The enzyme activities were assayed and expressed as described in METHODS.

|                | Ala-Ala-Ala- | Gly-Gly-Gly- | Phe-Gly-Gly- | Ala-Gly-Gly- | Bradykinin | Ala-Pro | Gly-Pro | Leu-Pro | Phe-Pro | Val-Pro | Pro-Ala |
|----------------|--------------|--------------|--------------|--------------|------------|---------|---------|---------|---------|---------|---------|
|                | HiGlyPhe    | Gly-Gly      |              |              |            |         |         |         |         |         |         |
| B. adolescentis| 13.38        | 1.26         | 4.19         | 0.33         | 0.94       | 1.58    | 0.21    | 0.58    | 0.73    | 1.92    | 11.96   |
| B. bifidum     | 92.11        | 1.59         | 27.29        | 0.69         | 40.75      | 3.44    | 0.34    | 1.54    | 2.79    | 2.90    | 10.88   |
| B. breve       | 83.85        | ND           | 31.72        | 1.59         | 28.87      | 3.44    | 0.49    | 2.25    | 2.55    | 3.58    | 5.50    |
| B. infantis    | 74.01        | ND           | 23.34        | 0.41         | 36.88      | 3.83    | 0.14    | 2.15    | 2.57    | 3.86    | 11.17   |
| B. longum      | 72.51        | ND           | 31.27        | 0.94         | 26.15      | 5.86    | 0.86    | 5.45    | 6.64    | 7.36    | 11.59   |

|                 | Pro-Gly | Pro-Leu | Pro-Tyr | Pro-Val | Ala-2-NA | Arg-2-NA | Glu-2-NA | Gly-2-NA | Leu-2-NA | Phe-2-NA | Pro-2-NA |
|------------------|---------|---------|---------|---------|----------|----------|----------|----------|----------|----------|----------|
| B. adolescentis  | 9.15    | 8.54    | 6.12    | 9.07    | 1.19     | 12.41    | 0.18     | 1.21     | 1.43     | 48.51    |
| B. bifidum       | 6.34    | 7.76    | 5.35    | 8.03    | 6.44     | 3.50     | 0.20     | 3.41     | 8.19     | 17.44    |
| B. breve         | 2.55    | 7.54    | 5.58    | 5.80    | 6.54     | 2.01     | 0.21     | 6.76     | 6.30     | 12.77    |
| B. infantis      | 8.50    | 8.13    | 5.46    | 8.60    | 7.99     | 2.57     | 0.09     | 0.37     | 3.80     | 8.32     | 38.15    |
| B. longum        | 8.84    | 8.29    | 5.57    | 8.94    | 7.31     | 2.15     | 0.12     | 0.34     | 4.48     | 8.80     | 38.32    |
Fig. 2. Aminopeptidase activities of the fractions fractionated by Disc-PAGE. The enzyme activities were assayed and expressed as described in METHODS.
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other strains, while those of the residual four strains were similar to each other. Inter-substrate analysis was also carried out. Synthetic substrates with a mutual correlation coefficient higher than 0.7 were classified as one group. Five groups were established (data not shown). All peptides of the X-Pro type and cbz-X-X type were included in cluster 2 and those of Pro-X type and Pro-2-NA type in cluster 4. The other peptides were distributed irregularly in clusters 1 to 5.

Disc-PAGE

The aminopeptidases in CFE obtained from those five strains of bifidobacteria were separated by Disc-PAGE. Figure 2 shows the results of an analysis of the aminopeptidase activity of each of the fractions extracted from these sliced gels against 14 kinds of synthetic substrates.

All of these five strains gave three active fractions, with only a slight difference in mobility. Active fraction A with a mobility of 0.44 to 0.46 hydrolyzed all the peptides except for Ala-Pro and Pro-2-NA types. Active fraction B with a mobility of 0.31 to 0.34 hydrolyzed only Ala-Pro type specifically. Active fraction C with a mobility of 0.16 to 0.19 showed hydrolyzing activity only against Pro-Ala and Pro-2-NA types. Pro-Ala type peptide was hydrolyzed by active fractions A and C.

DISCUSSION

Our experiments indicated that several kinds of aminopeptidases and carboxypeptidase were present in the CFE obtained from five strains of different species of bifidobacteria.

Bifidobacteria are classified into several species mainly according to their fermentative characteristics, but CFE obtained from five strains of different species subjected to the present study contained exopeptidases of similar profiles (Fig. 1). The results shown in Table 1 and Fig. 2 suggested that there are at least three kinds of aminopeptidases: (1) aminopeptidase with a broad substrate specificity (active fraction A may contain plural enzymes with close electrophoretic mobilities), (2) aminopeptidase which hydrolyzes X-Pro type dipeptides specifically (fraction B) and (3) aminopeptidase which hydrolyzes Pro-X type dipeptides specifically (fraction C). CFE also contained carboxypeptidase which hydrolyzes cbz-X-X type peptides.

These exopeptidases presumably function also in the metabolism of bifidobacteria which always occur in the intestinal canal of humans. Exopeptidase(s) present on the superficial layer of the bacterial cell may be thought to hydrolyze such nitrogen compounds as protein and peptides and to function in the uptake of these hydrolyzates by the bacterial body (a human, the host, may utilize part of them). Exopeptidase(s) on the inside of the bacterial body are presumably involved in the turnover of protein within the bacterial body.

Escherichia coli, which is the main bacteria present in the human intestine as well as bifidobacteria, contained dipeptidase(8), tripeptidase(9), aminopeptidase
N(10) and others (5, 11–14). These exopeptidases may function in a manner similar to that of the enzymes of bifidobacteria.

The present paper, which clarified the profiles and the homology of exopeptidases present in bifidobacteria, is thought to form the basis of the elucidation of the physiological function of bifidobacteria.

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