CHROMBIO. 3295

DETERMINATION OF β-ASPARTYLPEPTIDASE ACTIVITY IN HUMAN FAECES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING PRE-COLUMN DERIVATIZATION WITH PHENYL ISOTHIOCYANATE

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SUMMARY

Bacterial enzymes are responsible for degradation of β-aspartyl peptides in the intestinal tract. These peptides, especially the dipeptide β-aspartylglycine, are useful as indicators of an impaired anaerobic intestinal microflora of antibiotic-treated patients. A method to separate the dipeptides β-aspartylalanine, β-aspartylglutamine, β-aspartylglycine and β-aspartylserine, using reversed-phase high-performance liquid chromatography and pre-column derivatization with phenyl isothiocyanate, was developed. This method was used to determine β-aspartylpeptidase activity in faecal supernatants of healthy human volunteers and antibiotic-treated patients with β-aspartylglycine as substrate. This activity was absent in the antibiotic-treated group, while in individuals with an intact intestinal flora it ranged from 16 to 100% degradation per 18 h. In addition, it was found that faecal enzyme preparations cleaved β-aspartylglycine at a much lower rate than the other β-aspartyl peptides.

INTRODUCTION

The dipeptide β-aspartylglycine (β-Asp-Gly) is detectable in faecal supernatants of germ-free mice and antibiotic-treated patients [1—5]. It was shown that this dipeptide is a useful indicator of a substantial decrease of the number of bacteria in the intestinal tract [4, 5]. The formation of α-Asp bonds in the amino acid sequence of a protein occurs according to the scheme in Fig. 1. α-Asp and α-Asn cyclize to the cyclic imide form and are converted into β-Asp. α-Asn-Gly is converted ten times faster into β-Asp-Gly than is α-Asp-Gly [6]. Bovine ribonuclease is an example of a protein with an Asn-Gly sequence, and studies by Haley and Corcoran [7] have shown that extensive sequential degradation of ribonuclease with a number of proteases results in a mixture of free
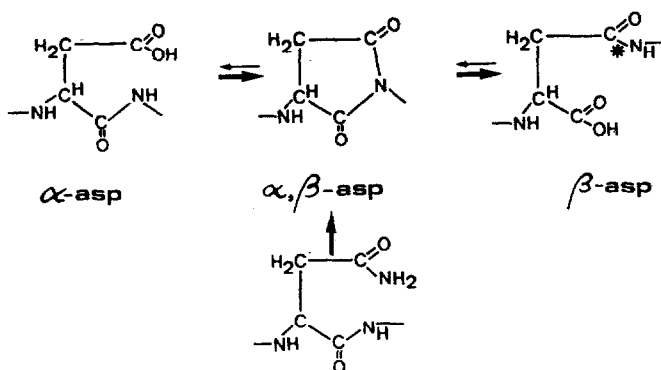
Fig. 1. Conversion scheme of α-aspartyl (α-Asp) via the cyclic imide, α,β-aspartyl (α,β-Asp) into β-aspartyl (β-Asp) and of α-asparaginyl (α-Asn) via the cyclic imide into β-aspartyl (β-Asp). The peptide bond to be cleaved by bacterial enzymes is indicated with an asterisk.

amino acids and β-Asp peptides. The β-Asp linkage was formed spontaneously and non-enzymically in fragments liberated during digestion. A similar phenomenon occurred during amino acid sequence studies on *Pseudomonas* cytochrome c-551, which contains Asp-Gly at residues 50—51. In proteolytic digests of this protein, β-Asp peptides as well as cyclic imide forms (α, β) of Asp peptides were encountered [8]. The accumulation of β-Asp peptides in the intestinal tract is the result of a combination of host-derived proteolytic activity and the absence of a special type of bacterial enzymatic activity. Because of their specific peptide bond, β-Asp peptides cannot be cleaved by host-derived enzymes, but can only be degraded by bacterial enzymes, which we shall call β-Asp peptidases.

In faecal supernatants of germ-free mice and totally decontaminated patients, β-Asp-Gly is generally present in much higher concentrations than e.g. β-Asp-Ala, β-Asp-Gln and β-Asp-Ser. To determine these β-Asp peptides in faecal supernatants, high-voltage paper electrophoresis (HVPE), followed by ninhydrin staining, appeared to be the most suitable method [9], but it is a semiquantitative technique with a detection level of ca. 1 nmol. High-performance liquid chromatography (HPLC), with pre-column derivatization [10, 11] of small amounts of β-Asp peptides, provides a system for a sensitive assay of β-Asp peptidase activity in faecal supernatants. β-Asp peptides are derivatized with phenyl isothiocyanate (PITC) and the resulting phenylthiocarbamyl (PTC) derivatives can easily be resolved with a non-gradient system. This HPLC system provides a way to determine the reason for the specific accumulation of β-Asp-Gly in the faeces of patients with an impaired intestinal bacterial flora.

EXPERIMENTAL

Materials

β-L-Asp-Gly was obtained from Serva (Heidelberg, F.R.G.), and β-L-Asp-L-Ala from Bachem (Bubendorf, Switzerland). PITC was sequenal-grade from Rathburn (Walkerburn, U.K.), stored at —20°C, and triethylamine (TEA) was purchased from Pierce (Rockford, IL, U.S.A.). Acetonitrile was HPLC-grade

from Alltech (Deerfield, IL, U.S.A.) and all other chemicals were of the purest grade commercially available. Analytical HVPE on Whatman 3 MM paper at pH 3.5 showed that commercially available β-Asp-Gly and β-Asp-Ala were in the β-form (blue colour with ninhydrin after heating above 120°C), and no longer in the cyclic imide form, as was found for previous batches of β-Asp-Gly [9]. Two additional β-Asp peptides were isolated from 1 ml of pooled faecal supernatant of totally decontaminated patients, by preparative HVPE as described by Ambler [8]. Amino acid analysis resulted in Asp and Ser and in Asp and Glu. Acid hydrolysis of β-Asp-Gln and β-Asp-Glu would result in the same amino acid composition: one Asp and one Glu. However, after HVPE the β-Asp-Glx spot was slightly less negative than β-Asp-Gly, which indicated that we were dealing with β-Asp-Gln.

Faecal supernatants

Faecal suspensions (25%, w/v) in water, containing 0.1% Triton X-100, were prepared by homogenizing the faeces with a glass-rod. After centrifugation at 2000 g for 30 min, the supernatants were sonicated (6 min, 60 W) and centrifuged for 60 min at 100 000 g in a 50 Ti rotor in a Beckman L5-65 ultra-centrifuge.

β-Asp peptidase assay

Crude enzyme solutions were prepared by dialysing each faecal supernatant (0.5 ml) from thirteen healthy volunteers and two antibiotic-treated patients against water in dialysis tubing (MW cut-off 12 000) during 24 h at 4°C. After lyophilization, each sample was dissolved in 2 ml of phosphate-buffered saline (PBS), pH 7.2, at 0°C. It was found that the β-Asp peptidase activity was dependent on the presence of metal ions, i.e. Zn^{2+} and to a lesser extent Co^{2+}. Therefore the incubation was carried out in the presence of 0.02 M zinc sulphate. To 180 μl of enzyme solution, 25 μl of 0.5 mM β-Asp peptide was added. A 40-μl aliquot was stored immediately at −20°C (zero-time sample).

The faecal enzyme solutions were incubated with β-Asp-Gly and in some cases also with β-Asp-Ala at 37°C for 18 h. From the sample with the highest peptidase activity a larger amount of enzyme solution was prepared. Degradation of β-Asp-Gly, β-Asp-Ala, β-Asp-Ser and β-Asp-Gln during 2, 4, 8 and 24 h incubation was investigated with this enzyme preparation. All experiments were carried out in duplicate.

Pre-treatment of samples for HPLC

Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were used for pre-treatment of the samples. The cartridges were equilibrated before use with 60% acetonitrile, followed by 100% water. After dilution at 0°C of 40 μl of sample in 460 μl of water, containing 0.01 mM β-Asp-Ala or β-Asp-Gly as internal standard, the solution was mixed and immediately applied to a Sep-Pak cartridge. When the Sep-Pak C_{18} cartridges are washed with water, the relatively acidic β-Asp peptides are partly removed from the C_{18} resin. Such losses could be reduced to less than 5% by pushing the solution containing β-Asp peptidase through the cartridge using a syringe without any further washing. The acidic compounds, which include β-Asp peptides, were

completely removed with 750 μl of 12% acetonitrile. For re-use the cartridge was rinsed with three 1-ml volumes of 60% acetonitrile and eight 1-ml volumes of water. This procedure can be used to treat up to thirty samples with one cartridge. The purified compounds were freeze-dried and dissolved in 100 μl of ethanol–water–TEA (2:2:1), and 30 μl were dried under vacuum before derivatization.

The derivatization procedure was as described by Bidlingmeyer et al. [11], i.e. 20 min at ambient temperature with 20 μl of freshly made mixture of ethanol–water–TEA–PITC (7:1:1:1). The reagents were removed under vacuum in a heated Speed-Vac centrifuge (Savant, Hicksville, NY, U.S.A.).

Chromatography

The chromatographic system was made up of a solvent-delivery pump (Model 2150, LKB, Bromma, Sweden), attached to a sample loop injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.). The Pico-Tag column (Waters Assoc.) which is a reversed-phase column (150 × 3.9 mm I.D.), equipped with a direct-connect guard column (Alltech), was kept at 37°C in a water-bath and connected to an absorbance detector (Model 441, Waters Assoc.). The guard column (30 × 2.1 mm I.D.) was packed with 300 mg of Spherisorb ODS-2, with particle size 10 μm (Phase Separations, Norwalk, CT, U.S.A.).

Each sample was dissolved in 50 μl of buffer A (0.14 *M* sodium acetate, 0.05% TEA, titrated to pH 6.35 with glacial acetic acid) and 10–45 μl were injected. Buffer A and buffer B (acetonitrile–water, 3:2) were both purged with helium. An isocratic elution system afforded a satisfactory separation of glutamic acid from β-Asp-Gly (see Fig. 2). In order to clean the column with buffer B automatically we applied a computer-controlled valve system as previously described [12]. The system was operated isocratically with buffer A for 11 min, changed to 100% buffer B in 2 min and returned at the same rate to 0% buffer B after washing for 2 min.
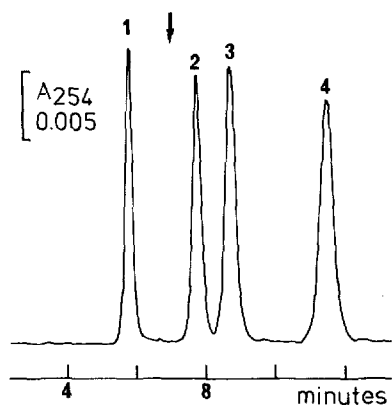

Fig. 2. Chromatogram of PTC derivatives from a reference solution containing 1 nmol each of aspartic acid (1), glutamic acid (2), β-aspartylglycine (3) and β-aspartylalanine (4). The arrow indicates the elution position of β-aspartylserine, and β-aspartylglutamine elutes at the same position as β-aspartylglycine. Chromatography is performed with a Pico-Tag column, connected to a guard column. Eluent, 0.14 *M* sodium acetate, 0.05% TEA, pH 6.35; flow-rate, 1.0 ml/min; detection, 254 nm at 0.05 a.u.f.s.

Amino acid analysis

Hydrolysates of β-Asp-Ser and β-Asp-Gln, which were isolated by HVPE (see *Materials*) were prepared in 50 × 6 mm I.D. tubes in the vacuum vial of Waters' Workstation [11]. Samples of ca. 1 μg of dipeptide were hydrolysed at 110°C for 24 h with 200 μl of constant-boiling hydrochloric acid at the bottom of the vacuum vial. The samples were cleared from acid residues by dissolving and drying twice with ethanol—water—TEA. Amino acid analysis with the Pico-Tag system was performed isocratically for 17 min to identify Asp, Glu, Ser and Gly.

RESULTS

β-Asp peptidase activities were determined as percentage degradation of β-Asp-Gly in faecal supernatants of thirteen healthy human volunteers and two decontaminated patients. The results are summarized in Table I. β-Asp peptidase activities in freshly prepared faecal supernatants of the healthy volunteers varied from 16 to 100% (median value 55%) degradation of β-Asp-Gly after 18 h incubation at 37°C. In contrast to this, β-Asp-Gly could not be cleaved by the faecal enzyme preparations of the decontaminated patients. Next we shall address the observation that β-Asp-Gly in particular accumulates in the faeces whereas other β-Asp peptides are found only occasionally [9]. Even in faecal supernatants of decontaminated patients, β-Asp peptidase activities of 27 and 30% degradation of β-Asp-Ala per 18 h were determined, whereas β-Asp-Gly was not cleaved at all. A faecal sample preparation of the volunteer whose faecal supernatant showed the highest β-Asp peptidase activity was used for incubation with the dipeptides β-Asp-Ala, β-Asp-Gln, β-Asp-Gly and β-Asp-Ser. The results of these investigations are given in Fig. 3, showing

TABLE I

DEGRADATION OF β-ASPARTYLGLYCINE AFTER 18 h INCUBATION AT 37°C BY FAECAL ENZYME PREPARATIONS OF THIRTEEN HEALTHY HUMAN VOLUNTEERS AND TWO ANTIBIOTIC-TREATED PATIENTS

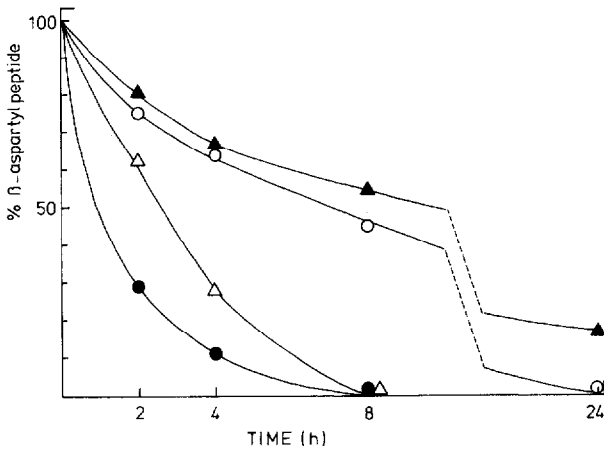| Volunteer	Degradation of β-aspartylglycine (%)
1	32
2	16
3	67
4	32
5	42
6	55
7	27
8	60
9	57
10	75
11	71
12	44
13	100
Patient 1	0
Patient 2	0

Fig. 3. Relative amounts of β-aspartylglycine (▲), β-aspartylglutamine (○), β-aspartylserine (△) and β-aspartylalanine (●) during incubation with faecal enzyme solution in PBS, pH 7.2 at 37°C. The enzyme solution was prepared from human faecal supernatants as described in the text.
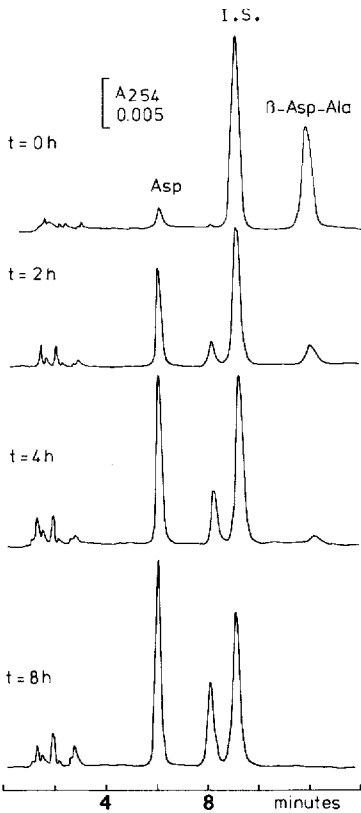

Fig. 4. Incubation of β-aspartylalanine with faecal enzyme of a healthy human volunteer, followed by chromatography with β-aspartylglycine as internal standard (I.S.). The peak between Asp and I.S. contains Glu originating from the faecal enzyme preparation. Samples were taken at 0, 2, 4 and 8 h. Chromatographic conditions were as in Fig. 2.

that β-Asp-Ala is cleaved at the highest rate and β-Asp-Gly at the lowest rate. An example of a series of chromatograms is given in Fig. 4, with decreasing amounts of β-Asp-Ala after 0, 2, 4 and 8 h incubation of β-Asp-Ala with faecal enzyme. In this particular case, and also when we studied the enzymatic degradation of β-Asp-Ser, β-Asp-Gly was used as internal standard.

β-Asp-Gln eluted at the same time as β-Asp-Gly, therefore the enzymatic degradation of β-Asp-Gln and β-Asp-Gly was studied using β-Asp-Ala as internal standard. The retention times of the relatively hydrophilic PTC-β-Asp peptides are short on the reversed-phase Pico-Tag column and, despite the isocratic elution conditions, they may vary from 11 to 12.5 min for β-Asp-Ala. Therefore, a reference mixture is advisable for adequate analysis of β-Asp peptides.

DISCUSSION

Direct determination of β-Asp-Gly in faeces is the most convenient way to determine bacterial β-Asp peptidase activity. Since the separation between β-Asp peptides and Asp and Glu is satisfactory (see Fig. 2), this was investigated with faecal samples from antibiotic-treated patients. However, the amount of Glu in such samples is generally more than 50 times larger than that of β-Asp-Gly, which prevents quantitative determination of β-Asp-Gly. Absence of β-Asp peptides is the result of a high in vivo β-Asp peptidase activity. Therefore, determination of the bacterial β-Asp peptidase activity in vitro can be used instead of a direct determination of β-Asp-Gly.

In healthy human volunteers with a complete bacterial intestinal flora, β-Asp-Gly degrading activity is present to a variable extent and such activity is undetectable in patients with an incomplete faecal flora. Therefore it is not surprising that β-Asp peptides accumulate in the faeces during strong suppression of the intestinal flora with antimicrobial agents.

Generally, in patients with an incomplete microflora, sufficient bacteria are present to produce β-Asp peptidases that eliminate β-Asp peptides such as β-Asp-Ala. Presumably, these β-Asp peptides, especially the specific peptide bonds, fit better into the active site cleft of the intestinal β-Asp peptidases. This in contrast to β-Asp-Gly, which is cleaved at a much lower rate.

The antagonistic effect of commensal microorganisms in the intestinal tract, which protect the host against opportunistic infections, is called colonization resistance (CR) [13]. Our results show that the earlier finding that β-Asp-Gly could serve as an indicator of decreased CR [2, 5, 9] is now supported by experimental evidence showing that it is this particular β-Asp peptide that is less susceptible to proteolytic degradation by β-Asp peptidase activity in faeces. This is the reason that β-Asp-Gly remains present in the faeces whenever the bacterial flora in the intestinal tract is affected, i.e. when a lower number of bacteria is present. β-Asp-Gly, therefore, may serve as an indicator of disappearance of certain components of the intestinal flora.

Intestinal β-Asp peptidases probably consist of a group of enzymes with similar activities and are produced by several bacterial species [5]. It was shown earlier [2] that after association of germ-free mice with 55 different strains of anaerobic bacteria, β-Asp-Gly became almost undetectable. This suggests that

at least a number of different strains of anaerobic bacteria produce β-Asp peptidases. This is also supported by the finding that, in patients treated with antibiotics, β-Asp-Gly was detectable when the normal concentration of *Bacteroides* sp., *Bifidobacterium* sp., *Eubacterium* sp. and *Clostridium* sp. was decreased [5]. Preliminary results show that a culture of *Bifidobacterium catenulatum* could degrade β-Asp-Gly to some extent [14]. The presence of β-Asp peptidase activity in *Bifidobacterium catenulatum* and other bacterial species will be investigated further.

ACKNOWLEDGEMENTS

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