***Bacillus stearothermophilus* Glyceraldehyde-3-phosphate Dehydrogenase as a Source of Angiotensin-converting Enzyme Inhibitors**

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The more potent inhibitory activity against angiotensin-converting enzyme (ACE) was excised from a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) preparation of *Bacillus stearothermophilus* by heating at 120°C in 1 M AcOH–20 mM HCl, as compared with GAPDH preparations of yeast and pig. Sufficient excision of *B. stearothermophilus* ACE inhibitors required a longer proteolysis time of 60 min. Two inhibitors were then purified by gel-permeation and reverse-phase chromatographies. One of the *B. stearothermophilus* ACE inhibitors, BG-1, was the GAPDH peptide 68–77 (Gly-Lys-Glu-Ile-Ile-Val-Lys-Ala-Glu-Arg, IC_{50} : 32 μ M). Another inhibitor, BG-2 (Gly-Lys-Met-Val-Lys-Val-Val-Ser-Trp-Tyr, IC_{50} : 6 μ M), corresponded to GAPDH peptide 304–313. These sequences were quite different from those of vertebrate GAPDH peptides and the venom peptide family with ACE inhibitory activity. BG-2 was found to be a non-competitive type inhibitor, differing from many natural peptide inhibitors. Thus, *B. stearothermophilus* GAPDH seemed to be a good source of new type ACE inhibitors, in addition to the advantages due to its thermophilic property.

The renin-angiotensin system is important in the regulation of blood pressure¹⁾ and angiotensin-converting enzyme (ACE; peptidyl dipeptide hydrolase, Ec 3.4.15.1) inhibitors have been of great value for their hypotensive effect through this system.²⁾ Further, recent studies suggest the participation of the renin-angiotensin system in functions unrelated to blood pressure regulation.^{3–5)} The search has continued for novel inhibitors of ACE from natural sources.⁶⁾ During our search for naturally occurring ACE inhibitors, we focused on a family of ACE inhibitory peptides excised from vertebrate glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) by acid-limited proteolysis,⁷⁾ as they were found to be unique, non-competitive inhibitors dif-

fering from many natural peptide inhibitors⁸⁾ and their sequences were quite different from those of the venom peptide family.

Since the amino acid sequences of GAPDH proteins are highly conserved among species,⁹⁾ ACE inhibitor may be produced from prokaryote enzymes. In this paper, we describe the significance of *B. stearothermophilus* GAPDH as a source of ACE inhibitors, compared with yeast enzyme, porcine enzyme, and bovine serum albumin (BSA).

Materials and Methods

Materials. *B. stearothermophilus* GAPDH (250 U/mg) prepared and highly purified in our laboratory¹⁰⁾ was used in this experiment. Baker's yeast was obtained from

Oriental Yeast Co. Ltd. (Osaka, Japan). After toluene plasmolysis, yeast GAPDH was extracted with 1 mM EDTA and purified by 60–80% saturated ammonium sulfate fractionation¹¹⁾ and by successive column chromatographies on DEAE-Sephadex¹²⁾ and *N*⁶-(*C*⁶-amino-hexyl)adenosine monophosphate-Sepharose 4B¹³⁾ (Pharmacia Fine Chemicals, Sweden). The purified yeast GAPDH preparation was finally eluted from the affinity column with 1 mM NAD⁺ and its specific activity was 150 U/mg protein. Pig muscle GAPDH (165 U/mg), rabbit lung ACE, and BSA were purchased from Sigma Chemical Co. (MO, U.S.A). Bovine lung ACE was prepared as described previously.¹⁴⁾ Hippuryl(Hip)-His-Leu was obtained from the Protein Research Foundation (Osaka, Japan), Asahipak GS-320 from Asahikasei Ind. Ltd. (Kanagawa, Japan), and Develosil ODS-7 from Nomura Chemical Ltd. (Aichi, Japan). All other reagents and solvents were obtained from Nakalai Tesque Ltd. (Kyoto, Japan).

ACE assay. The activity of ACE was assayed both by spectrophotometric and fluorometric measurements of the rate of formation of hippuric acid from Hip-His-Leu, by the method of Cheung and Cushman.¹⁵⁾ For either assay method, incubation was done at 37°C for 30 min in a final volume of 0.25 ml, which contained 100 mM potassium phosphate buffer (pH 8.3), 300 mM NaCl, enzyme (2 mU) and 2.5 mM Hip-His-Leu. The enzyme was rabbit or bovine lung ACE preparation. ACE-inhibitory activity was assayed in the presence or absence of inhibitor. For kinetic studies, in which concentrations of substrate, as well as inhibitor, were varied, each activity measurement was based on incubations of 15 min using a rabbit lung ACE preparation by fluorometric measurement. In other experiments, the inhibitor was preincubated with several concentrations of ZnCl₂ for 5 min at 37°C before the enzyme (rabbit ACE) reaction was started. Controls containing enzyme, substrate, and ZnCl₂ were run.¹⁶⁾ The activity was assayed by the fluorometric method.

Sequence analysis. Sequence analysis of peptides was done by stepwise Edman degradation using a gas-phase automated sequenator (Applied Biosystems, model 477A), coupled with HPLC identification of the resulting phenylthiohydantoin (PTH)-amino acid.

Production of ACE inhibitors. The procedures were essentially those used in extracting and fractionating the inhibitor from tuna muscle previously described,¹⁴⁾ except for the proteolysis time. Briefly, GAPDH, approximately 40 mg, was suspended in 20 ml of 1 M AcOH–20 mM HCl and autoclaved at 120°C for 5, 20, 60, or 120 min. After cooling, the extract was put into Sep-pak C₁₈ cartridges (Waters), and the cartridges were washed with 4% AcOH and eluted with 15% CH₃CN. The 15% CH₃CN fraction was referred to as Fr.I. Subsequent purifications of *B. stearotherophilus* Fr.I were done by HPLC.

Results

Untreated GAPDH preparations and BSA had no ACE-inhibitory activity even at a concentration of 100 μM. The time-dependent change in ACE-inhibitory activity of Fr.I during acid limited proteolysis of GAPDHs is illustrated in Fig. 1. In pig Fr.I, the inhibitory activity of about 10% was excised by heating for 5 to 60 min. Yeast Fr.I showed no activity up to 20 min, then the inhibitory activity (reaching about 40% at 120 min) appeared with the prolongation of proteolysis time. *B. stearotherophilus* Fr.I also showed no activity up to 20 min, but clearly had potent inhibitory activity of about 66% after proteolysis for 60 min and 120 min. BSA Fr.I did not have any activities. Thus, *B. stearotherophilus* GAPDH produced the most potent ACE-inhibitory activity under these proteolytic conditions.

Active components for ACE inhibition in Fr.I of *B. stearotherophilus* were purified by successive chromatographies on Asahipak GS-320 and Develosil ODS-7. When chromatographed by gel-permeation HPLC using Asahipak GS-320, Fr.I gave two peaks (BG-1 and -2) with ACE-inhibitory activity (Fig. 2). Following rechromatography on the same column, these active fractions were further pu-

Fig. 1. Time-dependent Change in ACE Inhibitory Activity of Fr. I during Acid-limited Proteolysis of GAPDHs.

(○), *B. stearotherophilus* GAPDH; (●), yeast GAPDH; (△), porcine GAPDH; (▲), BSA.

a) Fr. I prepared from 6.25 nmol each of untreated proteins at the indicated times was used for the assay.

rified by reverse-phase HPLC on a Develosil ODS-7 column (Fig. 3). Their activities were seen in a single peak each. Finally, rechromatography on the ODS column was necessary for removing a small amount of impurities, to give purified BG-1 and BG-2. The homogeneity was confirmed by another HPLC of the final preparations on Develosil ODS-7 and Asahipak GS-320 under the conditions shown in Figs. 2 and 3.

The results of automated Edman degradation for the two isolated peptides are shown in Table I with the ACE-inhibitory activity (IC_{50} value) and the yield from GAPDH protein. The more potent inhibitor, BG-2 (IC_{50} : $6 \mu M$

for bovine and rabbit lung ACEs), was a decapeptide consisted of Gly-Lys-Met-Val-Lys-Val-Val-Ser-Trp-Tyr, which corresponded to *B. stearotherophilus* GAPDH peptide 304–313.⁹⁾ The yield was 22.9 nmol from 1 μ mol of GAPDH. The amino acid sequence of another inhibitor, BG-1 (IC_{50} : $32 \mu M$ for bovine lung ACE), identified as Gly-Lys-Glu-Ile-Ile-Val-Lys-Ala-Glu-Arg, was that of GAPDH peptide 68–77.⁹⁾

Figure 4 shows Lineweaver–Burk plots for inhibition of rabbit lung ACE-catalyzed hydrolysis of Hip-His-Leu by BG-2. This peptide inhibited the enzyme activity non-competitively. The K_i from Dixon plots (data not shown) was $2.8 \mu M$.

When BG-2 ($6 \mu M$) was incubated without $ZnCl_2$ its ACE inhibitory activity was 54%. When 20 and $50 \mu M$ of $ZnCl_2$ were added, respective inhibitory activities were 42 and 48%. On the contrary, the inhibitory activity

Fig. 2. HPLC of Fr. I on Asahipak GS-320.

Column, 0.76×50 cm; solvent, 50 mM ammonium acetate (pH 6.7)/ CH_3CN (80/20); sample, Fr. I 1 mg in 100 μ l of solvent; flow rate, 1 ml/min. Arrows indicate peaks with ACE-inhibitory activity.

Table I. ACE INHIBITORS DERIVED FROM *B. stearotherophilus* GAPDH

Fraction	Amino acid sequence	IC_{50} (μM)	Yield (nmol) ^a
BG-1	Gly-Lys-Glu-Ile-Ile-Val-Lys-Ala-Glu-Arg	32	6.8
BG-2	Gly-Lys-Met-Val-Lys-Val-Val-Ser-Trp-Tyr	6	22.9

^a From 1 μ mol GAPDH.

Fig. 3. HPLC of Active Fractions from Asahipak GS-320 on Develosil ODS-7.

Column, 0.46×25 cm; solvent, 5–30% CH_3CN in 0.05% HCl (linear gradient); sample, concentrated active fraction in 100 μ l of 0.05% HCl; flow rate, 2 ml/min. Arrows indicate peaks with ACE-inhibitory activity.

Fig. 4. Lineweaver-Burk Plots of ACE-Inhibition by BG-2.

(70%) of EDTA ($3 \mu\text{M}$) was abolished completely by $20 \mu\text{M}$ of ZnCl_2 (data not shown). These data indicated that BG-2 did not have the chelating effect of the ACE active site zinc at all.

Discussion

Under acidic conditions at high temperatures, peptide bonds before and after Asp or Asn residues, especially Asp-Pro, are cleaved more rapidly than any other peptide bonds normally present in proteins.¹⁷⁾ The excision of ACE inhibitors (GAPDH peptides: Pro-X-Y-Ile-Lys-Trp-Gly-Asp, $\text{IC}_{50}^{7)}$: 10^{-6} – 10^{-5} M) from vertebrate GAPDH proteins occurred by acidic proteolysis at Asp-Pro (78–79 or 80–81) and Asp-Ala (86–87 or 88–89) which exposed to the exterior the dinucleotide binding domain of the dehydrogenase.¹⁸⁾ In *B. stearothermophilus* dehydrogenase, however, it seems difficult to produce the octapeptide sequence, Pro-Glu-Asn-Leu-Ala-Trp-Gly-Glu (79–86),⁹⁾ homologous to vertebrate ACE inhibitors, because the octapeptide is linked with the rest of the sequence by Glu-Ile (86–87).⁹⁾ Furthermore, the *B. stearothermophilus* GAPDH molecule retains its structural integrity, probably due to stable intramolecular interactions such as hydrophobic bonds and hydrogen bonds at temperatures up to 60 – 70°C ,¹⁹⁾ differing from the mesophilic enzymes. In fact, *B. stearothermophilus* GAPDH peptides prepared under the same proteolytic conditions (at 120°C in 1 M AcOH – 20 mM HCl) for 5

min as those used for vertebrate GAPDH proteins, did not show any ACE-inhibitory activity. Potent ACE inhibitory activity was induced first after heating for 60 min, suggesting the excisions at different cleavage sites from those of vertebrate GAPDH. The ACE-inhibitory activity induced from *B. stearothermophilus* GAPDH was much more potent than those from yeast and pig GAPDH proteins, as shown in Fig. 1. A highly purified GAPDH preparation of *B. stearothermophilus* is readily available in abundance because of its thermophilicity. Therefore, *B. stearothermophilus* GAPDH is a good source of natural ACE inhibitors with respect to mass production.

Two ACE inhibitors were purified from GAPDH peptides after acid-limited proteolysis, and their amino acid sequences were established and compared with the GAPDH peptide sequences reported.⁹⁾ One of the *B. stearothermophilus* ACE inhibitors, BG-1, was a GAPDH peptide, 68–77, excised by cleavages at Asn (probably *via* modification to Asp by acid)-Gly (67–68) and at Arg-Asn (77–78), and it was in just neighborhood of the sequence homologous to the vertebrate ACE inhibitors. Another inhibitor, BG-2, identified as Gly-Lys-Met-Val-Lys-Val-Val-Ser-Trp-Tyr, corresponded to GAPDH peptide 304–313 produced by cleavages at Asp-Gly (303–304) and Tyr-Asp (313–314).⁹⁾ GAPDH peptides containing the sequences homologous to vertebrate ACE inhibitors, however, did not release them under these conditions. The fold-

ing in the first domain (residues 0–149) of *B. stearothermophilus* GAPDH is a β - α - β pattern with a central sheet covered on both sides by helices.²⁰⁾ In the second domain the extensive anti-parallel β -sheet region forms a subunit interface with the equivalent region of the P-axis related subunit.²⁰⁾ BG-1 consisted chiefly of a β -sheet segment (residues 71–74) which lies close the exterior of the first folding, while the BG-2 segment was substantially one (304–311) of the β -sheets in the second domain and was buried in a gap between subunits. Anyway, the regions of BG-1 and BG-2 segments must be resistant to heating for 20 min under acidic conditions.

B. stearothermophilus ACE inhibitors did not share a common sequence with those of vertebrate GAPDH peptides and many natural peptides with ACE-inhibitory activity. Since ACE is a dipeptidyl carboxypeptidase, the C-terminal tripeptide residue of the inhibitor is predominant in its competitive binding to ACE. The terminal amino acid residue Pro or an aromatic amino acid is favorable for effective binding of peptide inhibitors to the active site of the enzyme,²¹⁾ and most of them contain Pro or aromatic amino acids as their C-terminal tripeptide residue.^{22,23)} A stronger and more reproducible inhibitor, BG-2, also had a C-terminal tripeptide residue of -Ser-Trp-Tyr. This peptide, however, was found to be a non-competitive type inhibitor, like tuna ACE inhibitor derived from GAPDH protein.

It has been reported that ACE also has tripeptidyl carboxypeptidase activity^{24,25)} and tripeptidyl aminopeptidase activity²⁵⁾ as well as that of a dipeptidyl carboxypeptidase. It is interesting how the inhibitors from GAPDHs affect these novel activities. Further investigation of inhibitors from GAPDHs may lead to development of more potent inhibitors and definition of the physiological roles of ACE.

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