Isolation and structure elucidation of an α-amylase inhibitor, AI-3688, from *Streptomyces aureofaciens*

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A novel polypeptide inhibitor, AI-3688, which acts upon human pancreatic α-amylase, was isolated from fermentation broth of *Streptomyces aureofaciens*. The purified peptide contains no unusual amino acids. Its M_r is 3936. The primary structure of AI-3688 was elucidated by automatic Edman degradation of the native or modified inhibitor. Two intramolecular cysteines form a disulphide bridge, thus creating a ring structure consisting of 17 amino acids. Strong sequence homology also exists to another microbial α-amylase inhibitor, tendamistat (HOE 467). This paper discusses the role of a common partial sequence, -Gln-Ser-Trp-Arg-Tyr-, present in the loop of both inhibitors as the active site of microbial peptide α-amylase inhibitors.

α-Amylase   *Microbial peptide*   *Amino acid sequence*   *Primary structure*

1. INTRODUCTION

Numerous microbial α-glucosidase-inhibiting substances of rather diversified chemical structure have been described [1,2]. A unique class is peptide inhibitors. Compounds of this type act specifically upon α-amylases [3–7]. Apparently they are not substrate analogues; however, their distinct mode of action is as yet unknown. A new peptide α-amylase inhibitor, AI-3688, has now been isolated from *Streptomyces aureofaciens*, FH 1656, DSM 2790 [8]. Elucidation of its primary structure should help to identify the effective partial sequence of the peptide inhibitors.

2. MATERIAL AND METHODS

2.1. *Fermentation of AI-3688*

A spore suspension of *S. aureofaciens* FH 1656, DSM 2790 strain was inoculated into 300 ml of a seed medium consisting of 4% dextrin, 1% glucose, 1% casein peptone, 0.4% corn steep liquor, 0.5% soybean meal, 0.8% $(NH_4)_2HPO_4$, pH 8.3, and incubated at 28°C for 24 h. 300 ml of the seed culture were added to 9 l of a production medium as above using a 12-l jar fermentor. Fermentation was carried out at 28°C under aeration of 420 l/h and agitation at 850 rpm. α-Amylase inhibitor production began 20 h after inoculation, then, gradually increasing, reached a maximum of 60 mg/l after 96 h.

2.2. *Purification procedure*

The fermentation broth (8 l) was centrifuged and the supernatant was adjusted to pH 4.9 and absorbed on a column (0.4 l) of resin Diaion HP-20. The column was washed with water and then with mixtures of water and isopropanol. The inhibitor was eluted with 25% isopropanol. Active fractions were collected and isopropanol was removed under reduced pressure. After concentration by ultrafiltration, the enriched solution was applied onto a DEAE-Sephadex column, equilibrated with phosphate buffer, pH 7.4. The inhibitor was eluted with 0.4 M NaCl using an NaCl gradient of 0–0.5 M. The fractions containing AI-3688 were again concentrated and desalted by ultrafiltration. Final purification was performed by chromatography on Sephadex G-50 fine using 30 mM phosphate buffer. α-Amylase inhibitor AI-3688 had a purity of 99% as indicated by gel electrophoresis.

2.3. Characterization of AI-3688

Analytical disc gel electrophoresis [9], determination of the isoelectric point and the formation of the complex with α-amylase (porcine pancreas) were performed as in [4]. The α-amylase-inhibiting activity was determined as described in [10].

2.4. Reduction with dithioerythritol

10 mg α-amylase inhibitor AI-3688 were dissolved in 5 ml of 8 M urea, mixed with 10 mg dithioerythritol in a closed vessel and allowed to stand for 24 h at room temperature. Samples were removed after 1, 2, 3 and 24 h and enzymatically examined.

2.5. Oxidation with performic acid [11]

5 ml performic acid were made by allowing 4.5 ml formic acid to react with 0.5 ml hydrogen peroxide (30%) for 30 min at room temperature. 20 mg AI-3688 were dissolved in 1 ml formic acid and oxidized for 3 h at 0°C with 2.5 ml performic acid. The sample was then diluted with water and evaporated to remove the formic acid. This step was repeated several times. Finally, the sample was lyophilized.

2.6. Amino acid analysis and sequence determination

Amino acid analysis was performed according to Spackman et al. [12]. Automatic Edman degradation in liquid phase with 1 mg Polybren and 0.1 M Quadrol was performed in a Beckman 890C sequencer equipped with a cold trap [13,14]. After drying, released amino acid thiazolinone was manually converted to phenylthiohydantoin with 1 N HCl and 0.01% thioethanol at 80°C for 10 min, dried and identified using HPLC on a Shandon Hypersil C-18 column with sodium acetate buffer (pH 5.3) and acetonitrile as organic modifier. Quantification was performed using N-leucine as internal standard.

3. RESULTS

3.1. Purification and chemical composition of AI-3688

During a screening program to obtain new kinds of microbial α-amylase inhibitors, the new producing strain S. aureofaciens FH1656 was found. Submerged, aerobic culturing of the strain using a culture medium containing starch resulted in the formation of the α-amylase inhibitor AI-3688. The method of isolation described above yielded a gel electrophoretically homogeneous material. Amino acid analysis revealed the AI-3688 to be composed solely of the natural amino acids; Lys, Met, His, Leu and Ile are absent. The isoelectric point is 4.2, the UV absorption corresponds to the protein spectra: $\lambda_{max} = 276$ nm ($A_{1\,cm}^{1\%} = 21$). The inhibitor is freely soluble in water and stable between pH 2–10 at room temperature.

3.2. Biochemical properties

In the photometric enzyme test, AI-3688 inhibits the α-amylase from porcine pancreas. The specific inhibitory action was determined to be 1.5×10^4 IU/mg. The effect of the inhibitor is 5-times lower against human salivary amylase in comparison to the pancreatic α-amylase, and almost ineffective against the α-amylase from Bacillus subtilus.

Maximal inhibition against the pancreatic α-amylase is attained after 5 min preincubation of the enzyme with AI-3688; inhibition is independent of the pH.

Mixtures of the pancreatic amylase with the inhibitor produce a complex which can be clearly recognized by gel electrophoresis. If the ratio of inhibitor to enzyme is increased to 12:1, none of the original components are detectable; a stoichiometric ratio of 1:1 exists.

Complex formation and α-amylase inhibition proceed unchanged even in the presence of 100-times the molar amount of p-chloromercuribenzoate [15]. Free -SH groups evidently play no part in the mechanism of α-amylase inhibition. On the other hand, the intact disulphide bridge of the inhibitor is necessary for its efficacy. Reduction of the bridge in 8 M urea solution leads to a time-dependent inactivation of AI-3688. After 1, 2, 3 and 24 h, 85, 75, 66 and 2%, respectively, of the implemented inhibitory efficacy are recovered.

3.3. The primary structure of the inhibitor AI-3688

Automatic Edman degradation of the native inhibitor and of the S-oxidized form was performed to determine the amino acid sequence (table 1). AI-3688 consists of 36 amino acids, arranged linearly, the 2 cysteines of the molecule creating a ring closure of the chain by formation of a disul-

Table 1

Sequence analysis of AI-3688

Step	Amino acid identified	Yield (nmol)
1	Ala	3.45
2	Thr	2.3
3	Gly	1.78
4	Ser[b]	–
5	Pro	3.1
6	Ala	2.7
7	Pro	2.5
8	Asp	1.75
9	Cys[a]	–
10	Val	2.0
11	Glu	1.6
12	Ser[b]	–
13	Phe	1.2
14	Gln	1.4
15	Ser[b]	–
16	Trp	0.38
17	Arg	0.98
18	Tyr	1.08
19	Thr	0.68
20	Asp	1.0
21	Val	0.86
22	Arg	0.68
23	Asn	1.05
24	Gly	0.41
25	Cys[a]	–
26	Ser[b]	–
27	Asp	0.56
28	Ala	0.53
29	Val	0.53
30	Thr[b]	–
31	Val	0.51
32	Val	0.6
33	Val	0.66
34	Gln	0.31
35	Tyr	0.30
36	Glu	0.33

[a] Identified as cysteic acid from oxidized material

[b] Identified as -Ser and as -Thr by the monitoring at 320 nm

phide bridge. Fig.1 shows the primary structure.

Among the proteins described to date, the amino acid sequence discovered is similar only to that of the microbial α-amylase inhibitor tendamistat (HOE-467). Sequence homology is more than 55%.

4. DISCUSSION

The mode of action of microbial proteins inhibiting α-amylase is not yet known. In particular, parts of these proteins responsible for their efficacy have not been discovered. The newly found inhibitor AI-3688 possesses very similar enzyme-inhibiting characteristics to tendamistat: (i) pancreatic α-amylase is inhibited, (ii) inhibition is independent of pH, (iii) a 1:1 inhibitor/enzyme complex is formed and (iv) the presence of the intact ring is necessary for inhibitory efficacy.

A comparison of the 2 primary structures reveals significant sequence homology from amino acid 3 to 39 of tendamistat, including the position and size of the ring. An alignment of the sequences is given in fig.1. Consequently, the inhibitorily effective part of HOE 467 seems to be associated with the 17-linked ring of the molecule or its environment. A Chou and Fasman calculation [16] to determine the chain formation shows that within the loops of AI-3688 and tendamistat respectively, the sequence -Gln-Ser-Trp-Arg-Tyr- is spatially exposed. In both molecules, the bridge-forming disulphides bend and stabilize the amino acid chains in such a way that this -Gln-Ser-Trp-Arg-Tyr- sequence can become exposed. However, in AI-3688 this sequence is located at the end of an α-helix, while in HOE 467 it is in a longer β-sheet structure. These data suggest that the partial sequence -Gln-Ser-Trp-Arg-Tyr- may correspond to the active site of the microbial protein-type α-amylase inhibitors.
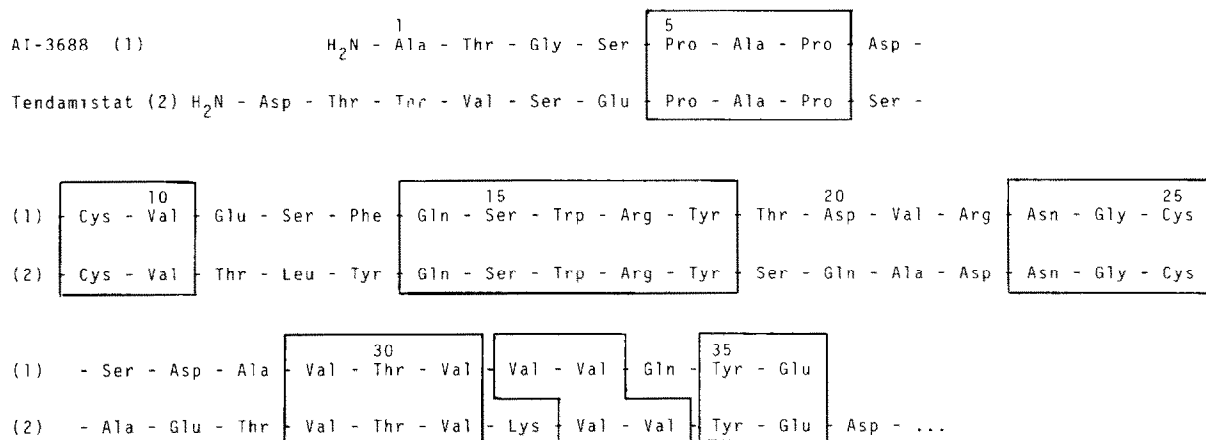
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                                          1                   5
AI-3688  (1)              H2N - Ala - Thr - Gly - Ser | Pro - Ala - Pro | Asp -

Tendamistat (2) H2N - Asp - Thr - Thr - Val - Ser - Glu | Pro - Ala - Pro | Ser -


            10                      15                    20                   25
(1) | Cys - Val | Glu - Ser - Phe | Gln - Ser - Trp - Arg - Tyr | Thr - Asp - Val - Arg | Asn - Gly - Cys |

(2) | Cys - Val | Thr - Leu - Tyr | Gln - Ser - Trp - Arg - Tyr | Ser - Gln - Ala - Asp | Asn - Gly - Cys |


                          30                               35
(1) - Ser - Asp - Ala | Val - Thr - Val | Val - Val | Gln - | Tyr - Glu |

(2) - Ala - Glu - Thr | Val - Thr - Val | Lys | Val - Val | Tyr - Glu | Asp - ...
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Fig.1. The amino acid sequence of AI-3688 and comparison with the primary structure of tendamistat (HOE 467). A disulphide bridge exists between the Cys (9) and Cys (25) of AI-3688 and between Cys (11) and Cys (27) of tendamistat.

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