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SPECIFICITY OF THE ACID PROTEASE FROM *MONASCUS KAOLIANG* TOWARDS THE B-CHAIN OF OXIDIZED INSULIN

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Summary

The proteolytic specificity of the acid protease from *Monascus kaoliang* has been investigated using the B-chain of performic acid-oxidized insulin as peptide substrate. Six splittings were detected after 1 h digestion and 12 splittings were found after 12 h incubation at 37°C, pH 4.8. The bonds most susceptible to the action of *M. kaoliang* acid protease were Phe(24)-Phe(25), Leu(15)-Tyr(16) and Tyr(16)-Leu(17). Among the acid proteases compared, the specificity of *M. kaoliang* acid protease on the B-chain of oxidized insulin is more closely related to that of penicillopepsin with which it has ten cleavage sites in common. *N*-Acetyl-L-phenylalanyl-L-3,5-diiodotyrosine, a synthetic substrate for pepsin, was resistant to the hydrolysis of *M. kaoliang* acid protease.

Introduction

Monascus kaoliang, the mould isolated from koji of kaoliang liquor, has been used in the fermentation industry for preparation of red rice wine, red Shao-Hsing wine, and native foods such as red soybean cheese, etc., for many thousands of years in China. It produces not only a lot of red, yellow and

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Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/147/69070/614 (1980) 607. The supplementary information includes: a table (Table I) listing amino acid composition, combining ratio, N-terminal residue, yield, and assigment of peptides produced by *Monascus kaoliang* acid protease action on the B-chain of oxidized insulin.

purple pigments, but also a large amount of hydrolytic enzymes [1]. The potential of the use of its enzymes as a feed additive has also been tried. Addition of 0.5% crude enzyme preparation to soybean meal significantly improves protein efficiency ratio and growth of weanling rats [2]. Recently, two forms of glucoamylase and an acid protease from *M. kaoliang* have been purified and partially characterized [3—5].

The purified *M. kaoling* protease is a glycoprotease possessing the nature of an acid protease with a pH optimum at about 3.0 toward heat-denatured casein [5]. The enzyme is inhibited by 1,2-epoxy-3-(*p*-nitrophenoxy)propane and pepstatin, resembling 'pepsin-like' acid proteases. It differs from pepsin in its insensitivity toward *p*-bromophenacyl bromide and biacetyl reagents. The glycoprotease nature of the *M. kaoliang* protease resembles *Mucor miehei* acid protease. The present investigation was undertaken to compare its proteolytic specificity with other acid proteases using the B-chain of oxidized insulin as the polypeptide substrate.

Materials and Methods

Materials. M. kaoliang acid protease was purified according to the procedure of Tsai et al. [5].

Beef crystalline insulin (lot 28C-0136, activity 26.8 I.U. per mg), Dowex 50-X2 (200-400 mesh, lot 96C-0301), dansyl chloride and dansyl amino acid were purchased from Sigma Chemical Co. Pyridine, formic acid, acetic acid, urea and 30% hydrogen peroxide were purchased from Merck Chemical Co. Constant-boiling hydrochloric acid in 1 ml ampoules, ninhydrin, stannous chloride and trinitrobenzenesulfonic acid were from Pierce Chemical Co. Polyamide layer sheets were obtained from Chen Hsin Tang Chemical Co., Ltd.

The B-chain of oxidized insulin was prepared by using the method of Craig et al. [6] and purified on Dowex 50-X2 equilibrated with 2.6 M formic acid. The B-chain was eluted from the resin with 1 M ammonia solution. Its purity was ascertained by N-terminal residue and amino acid composition analyses [7].

Digestion of the B-chain of oxidized insulin. 40 mg of the B-chain of oxidized insulin were dissolved in 0.5 ml of 50% formic acid and mixed with 10 ml of 0.55 M pyridine acetate buffer pH 4.8. After equilibration at 37° C, 2.0 ml of the solution were removed and lyophilized. To the remaining solution, 0.5 ml of 0.4% *M. kaoliang* acid protease in 0.55 M pyridine acetate buffer was added. The molar ratio of substrate to enzyme was 162 : 1. At intervals of 1 and 12 h, 4-ml aliquots of the reaction mixture were removed and freeze-dried. Also, at intervals, duplicate 0.1 ml aliquots were removed and placed into 1.0 ml 4% $NaHCO_3$, pH 8.5, for determination of the increase in amino groups by the trinitrobenzenesulfonic acid method [8]. The concentration of amino groups was calculated using a molar extinction coefficient of $1.3 \cdot 10^4\ M^{-1} \cdot cm^{-1}$.

Separation of digested peptides. Lyophilized digests from the enzymatic hydrolysis of the B-chain of oxidized insulin were dissolved in 0.5 ml of 0.2 M pyridine formate buffer, pH 3.1, and placed on top of a column of Dowex 50-X2 (0.9 × 60 cm) equilibrated with 0.2 M pyridine formate buffer, pH 3.1

at 40°C. Before use, the Dowex 50-X2 was purified as described by Hirs et al. [9] and Moore and Stein [10]. For elution of the peptides, the column was first washed with 80-ml 0.1 M pyridine formate buffer, followed by a linear buffer gradient increasing from 0.2 M pyridine formate buffer (280 ml), pH 3.1, to 3.0 M pyridine acetate buffer (280 ml), pH 5.0. The flow rate was 25 ml per h. 2.5-ml fractions were collected and analyzed for peptides by the ninhydrin method after alkaline hydrolysis [11,12].

Hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine by M. kaoliang acid protease. 0.1 ml of 0.1% enzyme solution was added to 1.0 ml of 0.16 mM substrate solution and incubated for a suitable time period. Then the solution was allowed to react with 0.5 ml ninhydrin solution in a boiling-water bath for 15 min. After cooling in cold water, 1 ml 50% ethanol was added. The absorbance of the solution at 570 nm was measured against a blank treated as above except 0.1 ml of water was added instead of the enzyme solution.

N-terminal residue and amino acid analysis. Amino acid analysis was performed according to the method of Spackmann et al. [13] on a JOEL-6AH automatic amino acid analyzer. Peptides were hydrolyzed with constant-boling HCl at 110°C for 24 h in evacuated sealted tubes. N-terminal amino acid was identified as dansyl amino acids on polyamide sheet [14,15].

Results

The rate of hydrolysis of the B-chain of oxidized insulin by M. kaoliang acid protease

The rate of hydrolysis in terms of the increase in amino groups determined by the trinitrobenzenesulfonic acid method is shown in Fig. 1. As shown, hydrolysis was rapid within the first 10 min with an average bond splitting of 1.40. This value doubled after approx. 4 h. At the end of 24 h, an average of 4.0 bonds were hydrolyzed.

Analysis of peptides from M. kaoliang acid protease digest of the B-chain of oxidized insulin

Dowex 50-X2 chromatographic separation of the peptides produced after

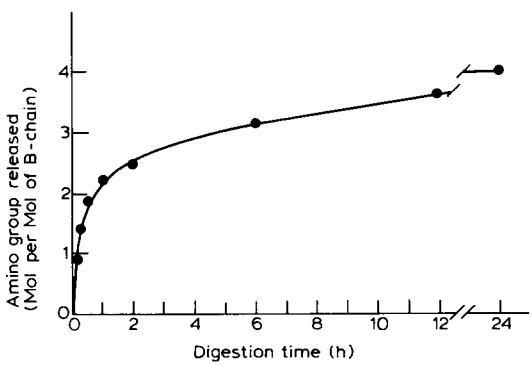

Fig. 1. The time course of liberation of free amino groups from digestion of the B-chain of oxidized insulin by *M. kaoliang* acid protease at 37°C, pH 4.8, and an enzyme to substrate molar ratio of 1 : 162.

1 and 12 h of incubation is shown in Fig. 2. The fractions indicated by the bars at the bottom of each diagram were pooled, lyophilized and hydrolyzed with constant-boiling HCl for amino acid analyses. The yield of each identified peptide was assumed to be proportional to the area it occupied in the elution diagram. It was calculated according to the equation:

$$A_i/A_t = Y_i \times (N_i/N_t)$$

where A_i, Y_i and N_i are the area, yield and number of amino acid residues of peak i, and A_t and N_t are the total area of peaks and number of residues of the B-chain, respectively. Different ninhydrin color yields from amino acid were not corrected.

The combining ratios of the amino acids and N-terminal residue obtained from each sample analyzed are shown in Table I (see footnote, p 607, data deposition). Identification based on the known sequence of the B-chain of oxidized insulin is also given in Table I. Ten peptides were determined after 1 h digestion and 17 peptides were identified after 12 h digestion. It was evident that short term hydrolysis produced three major splittings at Phe(24)-Phe(25), Leu(15)-Tyr(16) and Tyr(16)-Leu(17), to the extent of 55.0, 48.9 and 28.1%, respectively. The other detectable cleavages which occurred were of the bonds, Phe(1)-Val(2), Ala(14)-Leu(15) and Glu(13)-Ala(14). Peak No. 8 was a mixture of peptides 1-15 and 16-24. Peak No. 9 was a mixture of peptides 1-24 and 14-24. After 12 h hydrolysis, there were increases in bond splittings at Val(2)-Asn(3), Gln(4)-His(5), Ser(9)-His(10), His(10)-Leu(11), Val(18)-Cys(19) and Gly(23)-Phe(24). The N-terminal amino acid residue of peaks 17, 18, 20 and 24

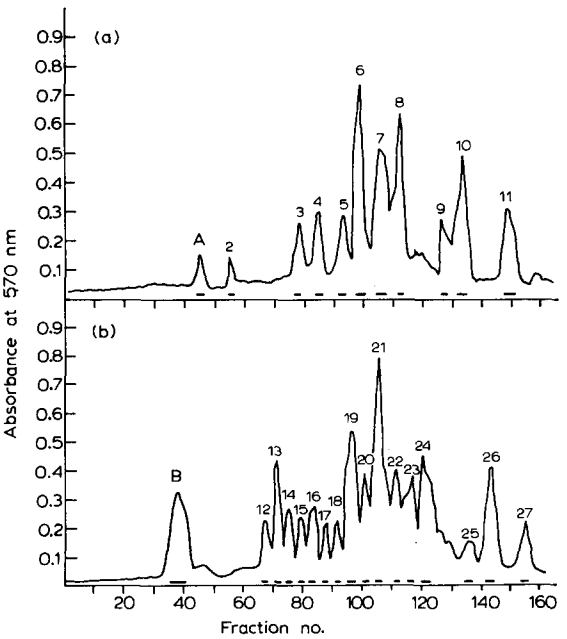

Fig. 2. Dowex 50-X2 chromatography of the peptides produced from digestion of the B-chain of oxidized insulin by *M. kaoliang* acid protease. (a) 1 h incubation, (b) 12 h incubation.

were not successfully determined. The assignments to peptides 3-9, 5-14, 3-10 and 3-14 were based on the results of amino acid analysis. Also the expected peptides 1-2, 14-18, 15-18, 17-18 and 16-18 were not detected. They may be lost during measurement.

Hydrolysis of N-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine by M. kaoliang acid protease

M. kaoliang acid protease was inactive toward the simple synthetic substrate for pepsin, *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine, after incubation for 1 h at 37° C.

Discussion

As shown in Table I and Fig. 3, the peptide bonds on the B-chain of oxidized insulin more susceptible to the action of *M. kaoliang* acid protease are mainly those involving amino acid residues with aliphatic or aromatic side chains. The enzyme preferentially splits the peptide bonds of Phe(24)-Phe(25), Leu(15)-Tyr(16) and Tyr(16)-Leu(17). Specificities of six other acid proteases, pepsin

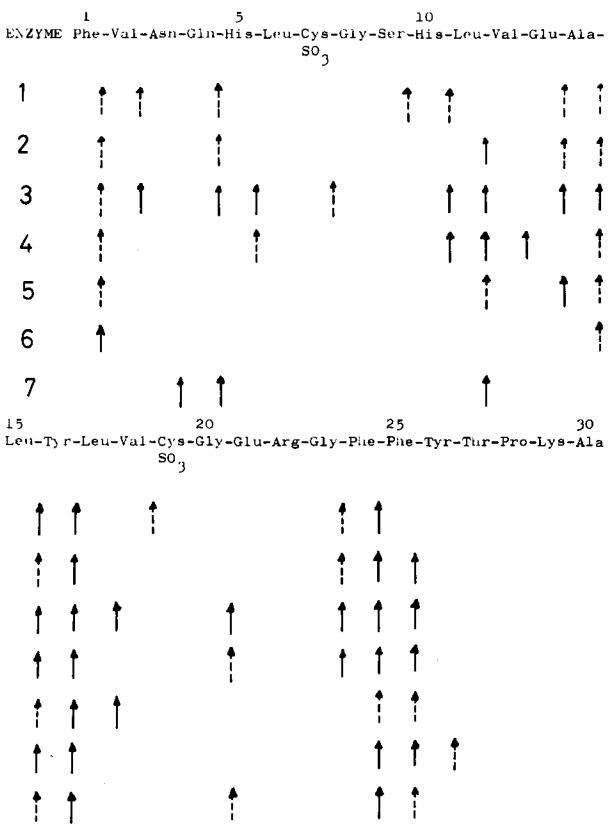

Fig. 3. A comparison of sites of cleavage of the B-chain of oxidized insulin by a number of acid proteases. Enzymes: (1) *M. kaoliang* acid protease, (2) pepsin, (3) penicillopepsin, (4) *Rhizopus* pepsin, (5) chymosin, (6) *M. miehei* acid protease, (7) *E. parasitica* acid protease.

[7], penicillopepsin [16], *Rhizopus* pepsin [17], chymosin [18], the acid proteases of *M. miehei* [19,20] and *Endothia parasitica* [21] on the same substrate are compared in Fig. 3. Apparently, the specificity of *M. kaoliang* acid protease differs more from those of chymosin and *M. miehei* and *E. parasitica* acid proteases than from those of pepsin, penicillopepsin and *Rhizopus* pepsin, in agreement with results of inhibition studies [5] which indicated that *M. kaoliang* acid protease is a 'pepsin-like' one. Interestingly, chymosin and *M. miehei* and *E. parasitica* enzymes all do not hydrolyze the bond, Gly(23)-Phe(24), which is hydrolyzed by pepsin, penicillopepsin and *Rhizopus* pepsin as shown in Fig. 3.

Among the acid proteases compared, *M. kaoliang* acid protease evidently is more closely related in its specificity to penicillopepsin with which it has ten cleavage sites in common. However, *M. kaoliang* acid peotease shows a slightly more restricted specificity than penicillopepsin. The appparent differences between specificity of *M. kaoliang* acid protease and other acid proteases on the B-chain of oxidized insulin could be related to the differences in secondary interaction in the binding cleft [22—24].

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