Characterization of the Complex between α_2 -Macroglobulin and a Serine Proteinase from *Bacillus natto*

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The stoichiometry of the reaction of bovine α_2 -macroglobulin (α_2 M) with a serine proteinase from *Bacillus natto* (BSP) showed that BSP bound to α_2 M in a molar ratio of about 2:1. The α_2 M-BSP complex hydrolyzed casein and succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosyl-4-methylcoumarin-7-amide (Suc-LLVY-MCA) 65 and 55% less, respectively, while the activity of fibrin hydrolysis was completely lost. The purified complex from DEAE-Toyopearl 650 S and Toyopearl HW-60 F chromatographies was homogeneous on polyacrylamide gel electrophoresis at pH 9.4. Immunoelectrophoresis of the α_2 M-BSP complex showed weak cross-reactivity with an antiserum to BSP (anti-BSP). The specificity of α_2 M-BSP complex to the acyl-LSTR-amide substrates Boc-LSTR-MCA and Boc-LSTR-pNA was partially changed. Enzymatic activity of the complex was completely abolished by chymostatin. Inactivation was observed when α_2 M-BSP complex was incubated with a 5-molar excess of *Streptomyces* subtilisin inhibitor (SSI) at pH 7.5 and 20°C. Interaction of α_2 M-BSP with ¹⁴C-succinyl-SSI was done at 20°C and at 4°C. Incorporation of ¹⁴C-succinyl-SSI with α_2 M-BSP was faster at 20°C than 4°C.

α_2 -Macroglobulin (α_2 M) is a plasma glycoprotein composed of four subunits, each of molecular weight 180,000 and linked through disulfide bonds.^{1~4)} It has been noted that α_2 M has the ability to bind all four proteinase classes: serine, cysteine, metallo, and carboxyl but not other enzymes or even inactive forms of these proteinases. α_2 M undergoes a conformational change on complex formation resulting in exposure of a specific receptor recognition site on the inhibitor.⁵⁾ Once bound, enzyme molecules are typically accessible to

substrates and inhibitors of low but not high molecular weight.⁶⁾ The following microbial serine proteinases have been shown to be either bound or inhibited by α_2 M: subtilopeptidase A and B, and neutral proteinases from *Staphylococcus aureus*, *Proteus vulgaris*, and *Trichophyton mentagrophytes*,³⁾ and *Serratia* 56 K protease.⁷⁾

Brinase, a serine proteinase from *Aspergillus oryzae*, administered intravenously in the doses used for therapeutic attempts did not circulate in free forms but was rapidly bound

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Abbreviations: α_2 M, α_2 -Macroglobulin; AMC, 7-amino-4-methylcoumarin; anti-BSP, antiserum to serine proteinase from *Bacillus natto*; Boc-LSTR-pNA; *t*-butoxycarbonyl-L-leucyl-L-seryl-L-threonyl-L-arginyl-*p*-nitroanilide; Boc-VLK-MCA, *t*-butoxycarbonyl-L-valyl-L-leucyl-L-lysyl-4-methylcoumarin-7-amide; BSP, serine proteinase from *Bacillus natto*; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SepII, serine proteinase from *Aspergillus sojae*; Suc-LLVY-MCA, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosyl-4-methylcoumarin-7-amide.

to α_2 M.⁸⁾ Larsson *et al.*⁸⁾ investigated the properties of α_2 M-brinase complex and assessed the activity of this complex against high-molecular-weight substrates. In addition, proteolytic enzymes have practical applications in tenderising meat. The preparation from fruit of papaya, papain, is rich in proteolytic activity and is now used industrially and domestically for tenderising meat. Extensive studies on meat proteolysis were done by Kang and Rice.⁹⁾ A serine proteinase from *Bacillus natto*,¹⁰⁾ a microorganism used to make a traditional Japanese fermented food, has been studied by us.¹¹⁾ But studies on its application have been scarce until now. To understand the mechanism underlying the inhibition of serine proteinases, a study was undertaken to examine the interaction of the proteinase with α_2 M as a safe antifibrinolytic agent or a meat tenderising agent.

Materials and Methods

Materials. Suc-LLVY-MCA, Boc-LSTR-MCA, Suc-AAPF-MCA, Boc-LSTR-pNA, and chymostatin were purchased from the Peptide Research Center, Inc., Mino, Osaka. Suc-AAPF-pNA and bovine serum albumin (fraction V) were supplied from Sigma, St Louis, U.S.A. α_2 M from bovine plasma was purchased from Boehringer Mannheim-Yamanouchi (Code No. 602442), Tokyo and anti-bovine whole serum from rabbit (Lot No. R867) was from ICN Immuno Biologicals, Lisle, IL, U.S.A. A mixture of cytochrome *c* oligomers was purchased from Oriental Kobo Kogyo Co., Ltd., Osaka. Phenylmethane sulfonyl fluoride was purchased from Nacalai Tesque Co., Ltd., Kyoto.

Serine proteinase from *Bacillus natto*. Extracellular serine proteinase (BSP) from *Bacillus natto* NC 2-1 ts 25 was purified by the previously described method.¹¹⁾

Assay of serine proteinase. Proteinase activity on milk casein at pH 7.5 was assayed as in our previous paper.¹¹⁾

Fluorometric assay. Suc-LLVY-MCA and Boc-LSTR-MCA were dissolved in a 25 mM solution of dimethyl sulfoxide (DMSO). The standard assay mixture contained 1.25 ml of 50 mM Na-phosphate buffer, pH 7.5, including 5 μ l of 25 mM substrate dissolved in DMSO, and 5 μ l of the enzyme preparation. A blank without enzyme was run in each series. Each mixture was incubated for 10 min at 30°C. The amount of 7-amino-4-methyl-coumarin (AMC) liberated was measured with a

Hitachi fluorescence spectrophotometer model F-3000. Excitation and emission wavelengths were 345 and 442 nm respectively. The instrument was calibrated daily so that the fluorescent intensity of a 1 μ M solution of AMC gave a full-scale response on a chart recorder.

Assay for *p*-nitroanilide substrates. Chromogenic activity on peptidyl-pNA was spectrophotometrically estimated with Hitachi 124 spectrophotometer at 380 nm. The standard assay mixture contained 2.5 ml of 50 mM Na-phosphate buffer, pH 7.5, including 10 μ l of 25 mM peptidyl-pNA, and 10 μ l BSP preparation. A blank without BSP was run in each series. Each mixture was incubated for 10 min at 30°C.

Assay of fibrin hydrolysis. One milliliter of 2% bovine fibrin suspended in 50 mM Na-phosphate buffer, pH 7.5, was combined with 50 μ l BSP at 30°C for 6 hr. The reaction was stopped by addition of 2 ml of 0.5 M Na-acetate buffer, pH 4.0, and filtered through a Toyo filter paper No. 2. Fibrin hydrolyzing activity is defined as the amount of enzyme yielding the color equivalent of 1 mol of tyrosine per second with Folin-Ciocalteu phenol reagent.

Purification of α_2 M and α_2 M-BSP complex. Purified α_2 M was prepared by repeated gel filtration on a Toyo-pearl HW 60 F column (1.5 cm $\times$ 90 cm). Elution was done with 50 mM Na-phosphate buffer, pH 7.5. The α_2 M-BSP complex was purified with a DEAE-Toyo-pearl 650 S column (1.5 cm $\times$ 15 cm) chromatography. Elution was done with an increasing concentration gradient of NaCl (0~0.5 M) in 10 mM Na-phosphate buffer, pH 7.5. Further purification was done by repeated gel filtration on a Toyo-pearl HW 60 F column (1.5 cm $\times$ 90 cm) with 50 mM Na-phosphate buffer, pH 7.5. Homogeneity of α_2 M-BSP complex was confirmed on polyacrylamide gel electrophoresis at pH 9.4.

Activity staining for Suc-LLVY-MCA. After electrophoresis, polyacrylamide gel was covered with a buffered Toyo filter paper No. 2 at pH 7.5 including 100 μ M Suc-LLVY-MCA for 10 min. The fluorescent AMC liberated from Suc-LLVY-MCA by α_2 M-BSP complex was detected with a Funakoshi transilluminator TL-33.

Protein concentration. The concentration of BSP was estimated by the method of Lowry,¹²⁾ using bovine serum albumin (fraction V) as a standard.

The protein concentration of bovine plasma α_2 M was estimated from absorption at 280 nm, with factor $A_{1\%}^{1\text{cm}} = 10.0$,¹³⁾ using a Hitachi 101 spectrophotometer.

Antisera. Anti-BSP was prepared from guinea pigs by the method described in the literature.¹⁴⁾

Immunoelectrophoresis. Immunoelectrophoresis¹⁴⁾ was

done with a Marysol KS-8415 apparatus (Yushima, Tokyo). Immuno-electrophoresis plates (75 mm $\times$ 49.5 mm) were coated with 1% (w/v) agarose (from Takara Shuzo Co., Ltd.) in buffer containing 0.38 M Tris-HCl buffer, pH 8.9. The capacity of each antigen well was 8 μ l and that of the serum trough 50 μ l. Electrophoresis was done for 1.5 hr at a current of 20 mA/plate in 25 mM Tris-glycine buffer, pH 8.3.

Antisera were placed into the plate slots and diffused at 20°C for 3 days. Immunoprecipitates were stained with Coomassie brilliant blue R-250 after being washed with 0.9% NaCl.

Polyacrylamide gel electrophoresis (PAGE). PAGE was done using 7.5% polyacrylamide gel in Tris buffer, pH 9.4, at 20 mA/plate at 4°C for 1.5 hr.

SDS-PAGE. SDS-PAGE was done with 7.5% acrylamide gel as described by Laemmli.¹⁵⁾ A mixture of cytochrome *c* oligomers was used as a molecular weight marker.

Protein staining. The protein was stained with Coomassie brilliant blue R-250 dissolved in 50% methanol-9.5% acetic acid, and destained in 5% methanol-9.5% acetic acid and 7% acetic acid as in our previous paper.¹⁶⁾

Kinetic parameter calculation. Kinetic parameters were calculated from Lineweaver-Burk plots.

Isoelectric point measurement. The isoelectric point was measured with a LKB Produkter AB isoelectric focusing apparatus LKB 8100.

Interaction of the α_2 M-BSP complex with SSI. Two hundred and fifty μ l (185 μ g) of the α_2 M-BSP complex were incubated with a 5-molar excess of SSI¹⁷⁾ and activity was assayed with Suc-LLVY-MCA at pH 7.5 and 4°C or 20°C.

Preparation of the α_2 M-BSP-SSI conjugate. SSI was added to the α_2 M-BSP complex, and incubated at 4°C overnight. The α_2 M-BSP-SSI conjugate was purified on Toyopearl HW 60 F column (1.5 cm $\times$ 90 cm). The α_2 M-BSP-SSI was concentrated with a collodion bag to 0.4 mg/ml. To each 40 μ l (16 μ g) of α_2 M-BSP-SSI conjugate was added 1.21 ml of 0.2 M phosphate buffer, pH 7.5. Residual activity of the mixture was measured with 10 mM Suc-LLVY-MCA as a substrate at 20°C.

Interaction of α_2 M-BSP complex with [1,4-¹⁴C]-Suc-SSI. Fifty μ Ci of ¹⁴C-succinic anhydride (Amersham Japan, Tokyo) was dissolved in 0.5 M Atkins and Pantin's buffer, pH 8.5, and then combined with 1.5 mg of SSI at 18°C for 10 min. Twenty μ l of succinic anhydride dissolved in dioxane was then added. After addition of three further

quantities of 20 μ l succinic anhydride, the reaction mixture was dialyzed against 50 mM Na-phosphate buffer, pH 7.5, to remove excess succinic anhydride. The specific radioactivity of ¹⁴C-Suc-SSI was 1.87 μ Ci/mg SSI.

α_2 M-BSP (0.4 mg/ml) was treated with 2.6 μ Ci ¹⁴C-Suc-SSI in 10 μ l at 20°C and 4°C; incorporation of ¹⁴C-SSI into the α_2 M-BSP complex was measured in each 200 μ l reaction mixture. The incorporation reaction was stopped with 5 μ l 40 mM PMSF. The α_2 M-BSP-¹⁴C-Suc-SSI conjugate was purified by PAGE using 5% polyacrylamide gel in Tris-HCl buffer (pH 8.9) at 17 mA/plate at 4°C for 17 hr.

Results

Characterization of BSP

Purified BSP was found to be homogeneous on an FPLC Superose 12 column. The SDS-PAGE pattern of the purified BSP is shown in Fig. 1. The enzyme behaved as a single component with a molecular weight of 30,000. The isoelectric point of BSP was 8.7. By the Ouchterlony double immunodiffusion technique, a good cross-reaction was achieved between BSP antiserum and BSP as shown in Fig. 2, but subtilisins Carlesberg and BPN' did

Fig. 1. SDS-PAGE of Purified BSP.

Lane 1, marker proteins of cytochrome *c* oligomers; Lane 2, BSP.

not crossreact.

Entrapment of BSP within α_2M

BSP was incubated with α_2M and then the

Fig. 2. Ouchterlony Double Immunodiffusion on Cross-Reactivity of Antiserum to BSP and Two Subtilisins, Carlsberg and BPN'.

Center well, antiserum to BSP; 1, BSP; 2, subtilisin Carlsberg; 3, subtilisin BPN'.

enzymatic activity of the mixture assayed with three substrates, casein, Suc-LLVY-MCA, and fibrin, in 50 mM Na-phosphate buffer, pH 7.5, at 30°C. As shown in Fig. 3, the proteolytic activity of BSP decreased proportionally with increasing amounts of α_2M , remaining con-

Fig. 3. Effects of α_2M on Enzymatic Activities of BSP.

Reaction mixtures were incubated with various molar amounts of α_2M in 50 mM Na-phosphate buffer, pH 7.5, at 30°C for 10 min, and residual enzymatic activities of the reaction mixtures were then measured with casein (○), Suc-LLVY-MCA (●), and fibrin (■).

Fig. 4. Polyacrylamide Gel Electrophoresis of α_2M -BSP Complex.

Each sample was put on a 7.5% polyacrylamide gel, and gel electrophoresis was done at 20 mA/plate and 4°C for 1.5 hr in Tris buffer, pH 9.4. A, proteins stained with Coomassie brilliant blue R-250; B, activity stained with Suc-LLVY-MCA; Lane 1, α_2M -BSP; lane 2, α_2M .

stant at 35% of the original caseinolytic activity even in the presence of excess α_2 M. Similar results were obtained with Suc-LLVY-MCA as a substrate, remaining constant at 45%. These results show that BSP stoichiometrically bound to α_2 M in a molar ratio of 2:1. When BSP was assayed with fibrin, activity remained constant at zero % of the original level in the presence of excess α_2 M.

The α_2 M-BSP complex was found to be homogeneous on PAGE. The R_f values of α_2 M-BSP complex and α_2 M on PAGE were 0.30 and 0.21, respectively (Fig. 4). Homogeneity of the complex was confirmed by immunoelectrophoresis (Fig. 5). Weak cross-reactivity of α_2 M-BSP with anti-BSP was observed on immunoelectrophoresis (2 in Fig. 5). However, when the antigenic activity of

α_2 M-BSP with anti-BSP was measured by the Ouchterlony immunodiffusion method, antigenicity of the α_2 M complex was found to be completely abolished. These results indicate that BSP is enclosed within the α_2 M molecule but the antigenic sites of BSP are not completely masked by the α_2 M molecule. Residual activity of the complex was completely abolished by chymostatin.¹⁸⁾

Specificity change of the α_2 M-BSP complex to Boc-LSTR-amides

BSP hydrolyzes the amide bond between amino acids and AMC in peptidyl-MCAs, Suc-LLVY-MCA, Suc-AAPF-MCA, Boc-LSTR-MCA, Boc-IEGR-MCA, Boc-EKK-MCA, and Boc-FSR-MCA. α_2 M-BSP also hydrolyzes Suc-LLVY-MCA, Suc-AAPF-MCA,

Fig. 5. Immunoelectrophoresis of α_2 M-BSP Complex.

Each sample was placed in the well of an agarose plate and electrophoresis was then done at 20 mA/plate in 25 mM Tris-glycine buffer, pH 8.3, followed by immunodiffusion using anti-BSP or anti-bovine whole serum for 3 days at 20°C. The immunoprecipitates were stained with Coomassie brilliant blue R-250 after being washed with 0.9% NaCl. Lane 1, α_2 M; Lane 2, the α_2 M-BSP; Lane 3, BSP.

Table 1. KINETIC PARAMETERS OF α_2 M-BSP COMPLEX TOWARDS SMALL FLUOROGENIC AND CHROMOGENIC SUBSTRATES AT pH 7.5

Substrate	α_2 M-BSP			BSP		
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
Suc-LLVY-MCA	0.02	0.10	5	0.01	0.14	14
Boc-LSTR-MCA	0.01	0.10	10	0.07	0.08	1
Suc-AAPF-pNA	0.57	1.2	2	0.36	4.0	11
Boc-LSTR-pNA	0.10	0.3	3	0.14	0.8	6

Boc-LSTR-MCA, Boc-IEGR-MCA, Boc-EKK-MCA, and Boc-FSR-MCA. α_2 M-BSP complex had significantly higher relative activities for Boc-LSTR-MCA and Boc-IEGR-MCA hydrolysis than those of BSP.

BSP hydrolyzes the amide bond between amino acids and *p*-nitroaniline in the peptidyl-*p*NAs, Suc-AAPF-*p*NA, Suc-AAPL-*p*NA, Suc-AAVA-*p*NA, Boc-LSTR-*p*NA, Z-GGL-*p*NA, Tos-GPK-*p*NA, and Boc-VLGR-*p*NA. The complex also hydrolyzes Suc-AAPF-*p*NA, Suc-AAPL-*p*NA, Suc-AAVA-*p*NA, Boc-LSTR-*p*NA, Z-GGL-*p*NA, Tos-GPK-*p*NA, and Boc-VLGR-*p*NA. α_2 M-BSP had significantly higher relative activities for Boc-LSTR-*p*NA and Boc-VLGR-*p*NA hydrolysis than those of BSP.

Kinetic parameters, K_m and k_{cat} , on small synthetic substrates at pH 7.5 are shown in Table I.

The enzymatic activities of α_2 M-BSP are retained when Suc-LLVY-MCA and Suc-AAPF-*p*NA are used as substrates. Conversely, when Boc-LSTR-MCA was substrate, enzymatic activity increased about 10-fold higher than BSP. When Boc-LSTR-*p*NA was used as the substrate, α_2 M-BSP activity decreased to about 50% that of BSP, indicating that an effective catalytic efficiency of α_2 M-BSP complex may depend on the low K_m values of Boc-LSTR-MCA and Boc-LSTR-*p*NA hydrolysis, and that the specificity of α_2 M-BSP complex for acyl-LSTR-amide substrates is partially changed.

BSP molecule in the α_2 M-BSP complex

Residual activities of α_2 M-BSP complex were completely abolished by the inhibitors phenylmethane sulfonyl fluoride (PMSF) and chymostatin,¹⁸⁾ while about 19% of residual activity of the α_2 M-BSP complex was observed with proteinaceous *Streptomyces* subtilisin inhibitor (SSI),¹⁷⁾ a high molecular weight inhibitor of serine proteinases.

Inactivation of α_2 M-BSP complex with or without SSI at 20°C in 50 mM Na-phosphate buffer, pH 7.5, is shown in Fig. 6. α_2 M-BSP complex was completely stable at 20°C over

Fig. 6. Inactivation of α_2 M-BSP Complex with or without SSI at 20°C.

●, presence of SSI; ○, absence of SSI.

Fig. 7. Temperature Dependence of Inactivation-reaction of α_2 M-BSP Complex with ¹⁴C-SSI.

●, at 20°C; ○, at 4°C.

20 hr. When treated with SSI, cessation of activities was observed at 20°C with a first-order rate constant, k , of $1.1 \times 10^{-6} \text{ s}^{-1}$.

The rate of ¹⁴C-Suc-SSI into the α_2 M-BSP complex at 20°C was 5.1 dpm/hr, and the rate at 4°C was 2.4 dpm/hr (Fig. 7). Incorporation of ¹⁴C-Suc-SSI with α_2 M-BSP was stronger at 20°C than at 4°C. We concluded that our results showed interaction of the α_2 M-BSP with ¹⁴C-Suc-SSI to be temperature-dependent.

Discussion

BSP from *Bacillus natto* has an isoelectric

point of 8.7 and a molecular weight of 30,000. There is no cross-reaction between antiserum to BSP and the two subtilisins Carlsberg and BPN' (Fig. 2). According to the Ouchterlony immunodiffusion method BSP is a serine proteinase different from subtilisins Carlsberg and BPN'.

Many proteases of four different enzyme classes interact with α_2 M.¹⁻⁴⁾ Interaction of α_2 M with proteinases has been reviewed by Barrett and Starkey.^{2,4)} Our study showed that interaction of BSP with α_2 M occurred in a molar ratio of 2:1 (Fig. 3). A 2:1 molar ratio was also reported for subtilisinpeptidase A by Wicker and Dolovich.¹⁹⁾ We reported a 2:1 ratio for the binding of a serine proteinase (SepII) from *Aspergillus*,²⁰⁾ but equimolar ratios were reported for elastase²¹⁾ and plasmin.²²⁾ A stoichiometry of close to 2:1 was also reported for the reaction between trypsin or chymotrypsin and α_2 M by Björk *et al.*²³⁾ A proteinase from *Aspergillus oryzae*, which had been used in therapeutic attempts as a thrombolytic agent in arterial thrombosis, bound to α_2 M with a stoichiometry of 1.7~1.9 mol of enzyme/mol inhibitor.⁸⁾

We used two methods, Ouchterlony double immunodiffusion and immunoelectrophoresis, to identify the antigenicity of α_2 M-BSP complex to anti-BSP. Which the former, the antigenicity of the α_2 M-BSP complex to anti-BSP was found to be completely abolished, but weak antigenicity on immunoelectrophoresis was detected, as shown in Fig. 5. Of the two methods, the latter is more sensitive.

The proteolytic activities of BSP remained constant at 35 and 45% of original caseinolytic and Suc-LLVY-MCA hydrolase activities even in the presence of excess α_2 M (Fig. 3), while when fibrin was used as the substrate, activity remained constant at zero %. Retaining of enzymatic activity may be caused by steric hindrance between α_2 M-BSP complex and fibrin, an insoluble substrate. These observation indicate that α_2 M-BSP is not sufficient for thrombolytic action *in vivo*. In a study of tenderising meat, the interaction of a serine proteinase from *Aspergillus sojae* has been in-

vestigated by Tsuji *et al.*²⁴⁾ The results demonstrate that the proteinase was greatly effective in the degradation of microfibrils and collagen. The proteolytic activity of α_2 M-BSP complex against high-molecular-weight substrate most likely accounts for the meat tenderising effect of BSP *in vivo*.

Previous results show that BSP hydrolyzed the oxidized insulin B-chain primarily at the peptide bond, Leu¹⁵-Tyr¹⁶, secondarily at an other bond, Ser⁹-His¹⁰, and two other sites (Gln⁴-His⁵, and His⁵-Leu⁶) but not the C-terminal peptide, Tyr¹⁶-Ala³⁰.¹¹⁾

When α_2 M-BSP complex was used for the hydrolysis of small fluorogenic and chromogenic amide substrates, specificity of the complex was partially changed for Boc-LSTR-MCA and Boc-LSTR-pNA. Peptides which have arginine in the P₁ position are preferentially cleaved by BSP, demonstrating that the S₁-P₁ interaction for Boc-LSTR-amide substrates may be important in increasing enzyme-substrate affinity and turnover rate. It can be concluded that specificity of α_2 M-BSP complex to Boc-LSTR-amides was partially changed.

When α_2 M-BSP complex was treated with SSI at 20°C, a decrease of enzymatic activity was observed with the first order rate constant, *k*, of $1.1 \times 10^{-6} \text{ s}^{-1}$. Incorporation of ¹⁴C-Suc-SSI with α_2 M-BSP was stronger at 20°C than at 4°C. We concluded that interactions of α_2 M-BSP with ¹⁴C-SSI are temperature-dependent.

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