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A Potent Inhibitor of Pancreatic Serine Proteases from Chick Skeletal Muscle

Ok Mae Kim, Sung Soo Chung, Hye Gyeong Park, Joon Ho Choe*, Chin Ha Chung, and Doo Bong Ha

*Department of Zoology, College of Natural Sciences, Seoul National University, Seoul 151-742; *Department of biological Science and Engineering, KAIST, Taejŏn 305-701, Korea*

A Potent inhibitor of trypsin and other various serine proteases including chymotrypsin, elastase, kallikrein, plasmin and subtilisin, has been purified to homogeneity from chick skeletal muscle by conventional chromatographic procedures. The inhibitor has an apparent molecular weight of 66,000 dalton as determined by gel filtration. When the purified inhibitor was electrophoresed in the presence of sodium dodecyl sulfate, there appeared two protein bands having molecular weights of 66,000 and 64,000 dalton. The 64,000 dalton protein seems to be the product of 66,000 dalton protein by a limited proteolysis during the purification procedure or *in vivo*. Thus, it seems to consist of a single polypeptide. The inhibitor appeared to be glycoprotein and have an isoelectric point of 7.4. It contains relatively large amount (8.33 mole%) of cysteine residues.

KEY WORDS: Trypsin, Serine proteases, Inhibitor

Most cellular proteins are continuously degraded and synthesized, and the protein turnover is characterized by heterogeneous rates of degradation of cellular proteins. In mammalian cells, a large number of endogenous proteins are degraded by lysosomal proteases, such as cathepsin B, D, H and L (Dean, 1979; Grinde and Seglen, 1980; Shaw and Dean, 1980). Although lysosomes are considered to play an important role in intracellular protein breakdown, a second, nonlysosomal pathway has been emphasized to explain this heterogeneity (Dean, 1980; Hershko and Ciehanover, 1982). In muscle cells, 60% of the intracellular proteins are constituents of contractile myofilaments, and the average turnover rates of sarcoplasmic proteins and contractile proteins differ considerably (Bates and Millward, 1983). In addition, since there are relatively low lysosomal contents, such a nonlysosomal proteolytic pathway should be of great importance in a tissuelike muscle.

Various non-lysosomal proteases have been isolated in muscle, and these include Ca^{2+}-activated neutral proteases (Ishiura *et al.*, 1978), chymotrypsin-like protease (Kuo and Bhan, 1980; Sohar *et al.*, 1986), trypsin-like protease (Kay *et al.*, 1982a; Kay *et al.*, 1982b), high molecular weight protease (Dahlmann *et al.*, 1983b; Dahlmann *et al.*, 1985; Ismail and Gevers, 1983), insulin-degrading protease (Ryan and Duckworth, 1983), ATP-dependent protease (Ismail and Gevers, 1983), and hydrolase H (Okitani *et al.*, 1981). However, little is known about their physiological substrates and specific functions.

Commonly, wherever a protease particularly

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from mammalian cells is found in nature, there too exists a specific inhibitor of that enzyme to regulate its function. For determination of the roles that these proteases play in muscle protein catabolism and the regulation of such proteolytic activities *in vivo*, both proteases and their inhibitors should be studied.

In the present study, a proteinaceous inhibitor active against trypsin as well as various other serine proteases was purified to homogeneity to learn about its properties and therefore to clarify its physiological functions in the regulation of intracellular protein breakdown. Since this inhibitor is active only against serine-specific proteases, it was named as serine-protease-inhibitor (SPI).

Materials and Methods

Materials

N-benzoyl-DL-p-nitroanilide (BAPNA), trypsin, chymotrypsin, elastase, kallikrein, plasmin, subtilisin (type VII and VIII) thermolysin, and papain were purchased from Sigma. Sephacryl S-200, S-300 and Pharmalytes were also obtained from Sigma; DEAE-cellulose (DE-52) and CM-cellulose (CM-52) from Whatman; ^{125}I-protein A from New England Nuclear. [^{3}H]Casein was prepared as described by Rice and Means (1971). Other chemicals were of analytical grade.

Assays

The inhibitory activity of SPI against trypsin was assayed as described by Chung *et al.* (1983). One unit of inhibitor was defined as the amount that caused a 50% inhibition of the hydrolysis of BAPNA by 1 μg of trypsin in a final reaction volume of 1.0 ml. A typical reaction mixture contained 1 μg of trypsin, 1 mM BAPNA, 0.1 M Tris-HCl (pH 8.0), and 20 mM $CaCl_2$. Following the incubation for 30 min at 37°C, 0.5 ml of 30% (v/v) acetic acid was added to stop the reaction. The absorption of p-nitroanilide released during the incubation period was measured at 410 nm.

[^{3}H]Casein-degrading activities of various proteases were determined by measuring the radioactivity of the soluble products in 10% (w/v) trichloroacetic acid (TCA) as described by Goldberg *et al.* (1982). Reaction mixtures contained a proper amount of protease and 10 μg of [^{3}H]casein in a final volume of 0.1 ml. Incubations were performed at 37°C for 10 min.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to Laemli (1970) by using 8-17% (w/v) gradient slab gels. Isoelectric focusing of SPI in 5% polyacrylamide slab gel was carried out as in Tsuji *et al.* (1986).

Amino acid analysis

SPI (1.2 nmoles) was hydrolyzed in 6N HCl at 110°C for 24 hrs and 48 hrs in a sealed evacuated tube. The analyses were performed in a LKB 4151 α plus amino acid analyzer.

The concentration of free sulfhydryl groups was estimated by the modified procedure of Ellman (1959). The reaction mixture (0.94 ml) was prepared by adding the following reagents in order; 200 μg of SPI in 0.25 ml, 0.45 ml of 20% SDS, 0.2 ml of 0.15 M Tris-HCl (pH 8.0), and 0.04 ml of 15 mM 5,5′-dithio bis (2-nitrobenzoic acid) in 0.15 M Tris-HCl (pH 8.0). The absorbance at 412 nm was then measured in a spectrophotometer.

The concentration of tryptophan was estimated spectrophotometrically as described by Edelhoch (1967).

Results

Purification

All the purification steps were carried out at 4°C unless otherwise indicated. Crude extract was prepared by homogenizing for 3 min chick skeletal muscle (400 g) in 4 volumes of 20 mM Tris-HCl (pH 7.8) containing 0.1 mM EDTA using a Waring blendor. The homogenate was then centrifuged at 12,000 × g for 1 hr and the supernatant was filtered through filter papers (Whatman #2) to remove fat and cell debris. This filtrate was adjusted to pH 7.8 by adding 2.0 M Tris base and loaded onto a DEAE-cellulose column (5 × 22 cm) equilibrated with 20 mM Tris-HCl (pH 7.8). After washing the column extensively with the same

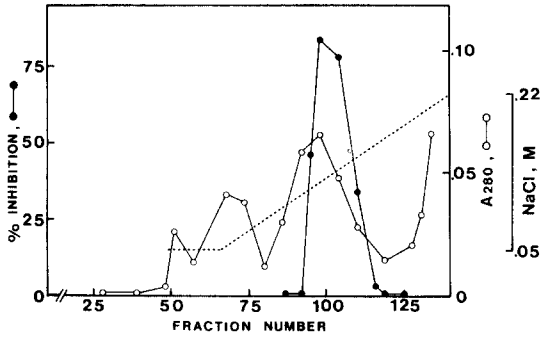

Fig. 1. The 2nd DEAE-cellulose chromatography. The proteins obtained from the 1st DEAE-cellulose step were fractionated by adding crystalline ammonium sulfate to 50% saturation, dialyzed against 20 mM Tris-HCl (pH 7.8), and loaded on a DEAE-cellulose column (2.2 × 9.0 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient of 0 to 0.12 M NaCl and fractions of 9 ml were collected at a flow rate of 100 ml/hr. The protein profile (○) was determined by the absorbance at 280 nm and the trypsin inhibitory activities (●) were assayed as described in "Materials and Methods".

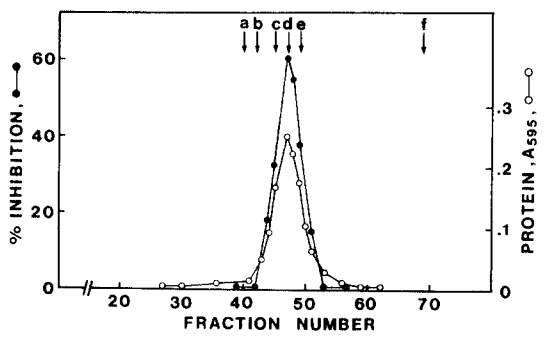

Fig. 2. Chromatography on Sephacryl S-200 column. Fractions of 65 to 72 from the Sephacryl S-300 step were pooled, concentrated, and loaded with 50 mM Tris-HCl (pH 7.8) containing 50 mM NaCl. Fractions of 1 ml were collected at a flow rate of 5 ml/hr. Aliquots (4 or 8 μl) of each fraction were assayed for trypsin inhibitory activity, and the protein amount was determined in 20 μl of each fraction by Braford method. The inhibitory activity was proportional to the amount of protein in each fraction. The arrows indicate where the marker proteins eluted: a, catalase (Mr. 240,000); b, alcohol dehydrogenase (150,000); c, hexokinase (100,000); d, bovine serum albumin (68,000); e, ovalbumin (44,000); f, trypsin (23,800).

buffer, the proteins were eluted with 0.1 M NaCl and collected at a flow rate of 500 ml/hr. The fractions showing the trypsin inhibitory activity were pooled, fractionated by adding solid ammonium sulfate to 50% saturation, and dialyzed against the same buffer. The dialyzed proteins were adsorbed to a DEAE-cellulose column (2.2 × 9.0 cm) equilibrated with the buffer and eluted with a linear gradient of 0 to 0.12 M NaCl at a flow rate of 100 ml/hr. As shown in Fig. 1, the peak of the inhibitory activity was eluted at approximately 0.06 M NaCl The active fractions were pooled, dialyzed against 10 mM sodium acetate (pH 4.5), and applied to a CM-cellulose column (1.5 × 11 cm) equilibrated with the acetate buffer. The proteins were eluted with a linear gradient of 0.05 to 0.22 M NaCl at a flow rate of 60 ml/hr. The peak of inhibitory activity eluted at about 0.13 M NaCl. The active fractions were pooled, adjusted to pH 7.8 as above, concentrated by ultrafiltration using an Amicon PM 10 membrane, and loaded on a Sephacryl S-300 column (2 × 78 cm) equilibrated with 50 mM Tris-HCl (pH 7.8) containing 50 mM NaCl. SPI obtained from the column was concentrated as above and loaded on a Sephacryl S-200 column (1.3 × 80 cm) equilibrated with the same buffer (Fig. 2). Active fractions were pooled and kept at 4°C for further use. The summary of the purification protocol is shown in Table 1.

Physical and chemical properties

Apparent molecular weight of SPI was determined to be 66,000 dalton using gel filtration on a Sephacryl S-200 column (Fig. 2). When the purified SPI was electrophoresed in a gradient polyacrylamide gel in the presence of SDS, there appeared two protein bands having molecular weight of 66,000 dalton and 64,000 dalton (Fig. 3). To determine if the one with lower molecular weight is a contaminating protein or if it is a limited degradative product of the larger polypeptide, the cleavage pattern of the proteins was analyzed by treating trypsin during the 2nd gel electrophoresis in the presence of SDS. The cleavage pattern of the larger protein is very much similar to that of the smaller protein (data not shown). Thus, it is likely that the 64,000 dalton protein is the product of the 66,000 dalton protein by a limited proteolysis that might have occurred

Table 1. Summary of purification procedures

Steps	Total Protein (mg)	Total Activity (Unit)	Specific Activity (Units/mg)	Recovery (%)	Fold Purification
Homogenate	8,688.0	5,688	0.7	100	1
DEAE-cellulose	994.0	4,819	4.9	.85	7
Ammonium Sulfate	427.5	3,800	8.9	67	14
DEAE-cellulose	253.8	3,327	13.9	59	20
CM-cellulose	13.5	2,600	192.6	46	292
Sephacryl S-300	3.4	2,212	650.6	39	997
Sephacryl S-200	1.7	1,439	846.5	25	1,283

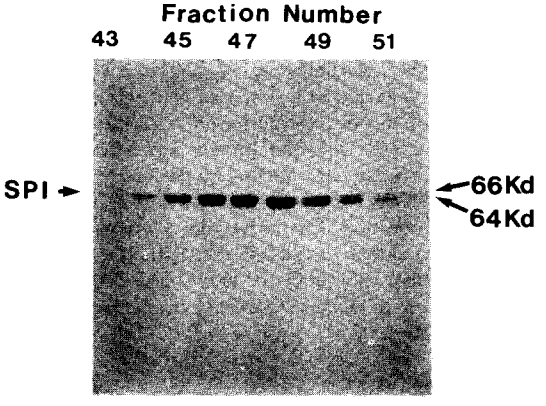

Fig. 3. SDS-polyacrylamide gel electrophoresis of SPI obtained from Sephacryl S-200 column. The fractions (from 43 to 52) obtained from Sephacryl S-200 column were subjected to SDS-polyacrylamide gel electrophoresis. The inhibitory activity was also proportional to the intensity of Coomassie-stained protein band shown. This result indicates that two proteins (66 and 64 kD SPI) have equal activities.

Table 2. Analysis of amino acid composition of SPI

Amino Acids	Mol %
Asx	11.87
Thr	7.00
Ser	7.66
Glx	10.33
Pro	6.00
Gly	9.33
Ala	6.00
Val	4.83
Half-Cys	8.33
Met	1.00
Ile	2.50
Leu	5.17
Tyr	3.17
Phe	1.50
Lys	5.66
His	3.17
Arg	5.83
Trp	0.66
ToTal	100.01

Asx: Asp+Asn, Glx: Glu+Gln

during the purification period or *in vivo*. Therefore, the isolation procedure used appears to yield a homogeneous preparation of SPI and the protein seems to consist of a single polypeptide.

SPI appeared to be glycoprotein because both proteins were stainable with Periodic Acid-Schiff's (PAS) reagents (Fig. 4). However, SPI did not bind to a concanavalin A-Sepharose column, suggesting that the carbohydrate chain of SPI contains a few α-D-glucose and α-D-mannose, if any. Because SPI diffused rather severely on 10% polyacrylamide gels perhaps due to the presence of carbohydrate residues, the gels prepared with concentration gradient were used throughout this study.

To determine the isoelectric point (pI) of SPI, 5% polyacrylamide gels containing Ampholytes were used for isoelectric focusing gel electrophoresis. SPI localized as a single band seen by staining with Coomassie blue and to the same position corresponding to pH 7.4 (data not shown). Thus, SPI appears to have a pI value of 7.4.

The amino acid composition of SPI is shown in Table 2. It is interesting that SPI contains relatively large amount (8.33 mole %) of cysteine residues

but no free sulfhydryl groups. Thus, all the cystein residues are likely to form disulfide bonds in three dimensional structure of SPI.

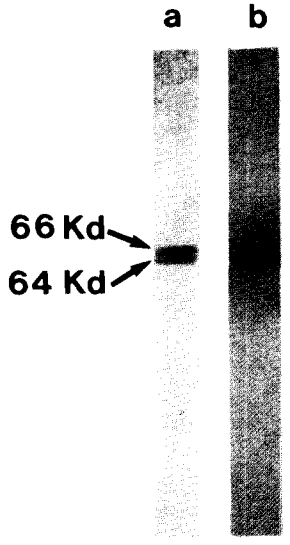

Fig. 4. PAS-stained SPI compared with Coomassie-stained SPI. a, SPI stained with Coomassie blue. b, SPI stained with Periodic Acid- Schiff's reagents. PAS (Periodic Acid, Schiff's reagent) staining was performed using the procedure described by Matthieu and Quarles (1971) with some modification.

Table 3. Effect of SPI on the activity of various proteases

Proteases(50 ng)	SPI(ng)	Inhibition(%)
Trypsin	50	38
	100	68
Chymotrypsin	50	23
	100	44
Elastase	50	60
	100	80
Kallikrein	50	84
	100	89
Plasmin	50	57
	100	65
Subtilisin VII	50	9
	100	38
Subtilisin VIII	50	16
	100	33
Thermolysin	50	0
	800	14
Papain	50	0
	800	9

The activities of various proteases were determined by using [^{3}H]casein as a substrate. Reaction mixture contained 50 ng of protease and 10 μg of [^{3}H]casein in a final volume of 0.1 ml as described in the text. Incubatons were performed at 37°C for 10 min. Similar data were obtained in at least three different experiments.

Effect of SPI on various proteases

The ability of SPI to inhibit other proteases besides trypsin was tested by incubating the inhibitor with them (Table 3). SPI strongly inhibited all mammalian serine proteases assayed, including chymotrypsin, elastase, kallikrein and plasmin. It also inhibited, but less effectively, subtilisin type VII and VIII. By contrast, the activity of thermolysin and papain was not affected by SPI. It neither reduced the activity of partially purified Ca^{2+}-activated neutral protease obtained from chick muscle. Therefore, SPI appears to inhibit rather specifically the serine proteases but not metallo- or sulfhydryl proteases.

DISCUSSION

A variety of protein inhibitors of serine proteases have been isolated from diverse sources such as *E. coli* (Chung *et al.*, 1983), legume seeds (Laskowski and Kato, 1980), avian eggs (Kato *et al.*, 1978), mammalian pancreas (Laskowski and Kato, 1980), blood plasma (Travis and Salvesen, 1983), mucus secretions (Smith and Johnson, 1985), parotid secretions (Thompson and Ohlsson, 1986), urine (Balduyck *et al.*, 1985), and muscle (Carney *et al.*, 1980; Kuehn *et al.*, 1984). These inhibitors have the common feature inactivating serine proteases such as trypsin, chymotrypsin and elastase but not sulfhydryl or metalloproteases. As some physical properties that they also share, although a number of inhibitors are excepted, such inhibitors have isoelectric points between pH 4 and 5 and tend to be stable at low pH and high temperature. Resistance to boiling and acid is atributed to the relatively high content of disulfide bridges.

Similarly to these inhibitors, SPI inhibits selectively the serine proteases but not the sulfhydryl or metalloproteases (Table 3). In addition, SPI is stable at pH 1 and at least for 5 min at 100°C (data not shown). However, unlikely to these in-

hibitors, SPI has a neutral isoelectric point (pH 7.4) and contains unusually high amounts of disulfide bonds. Assuming that a molecule of SPI consists of 600 amino acids since the size of SPI is estimated to be 66,000 including the molecular weight of its carbohydrate chain (s), it contains 8.33 mole % of half-cysteine residues (Table 2) which corresponds to 25 disulfide bridges. However, the precise number of disulfide bonds is unknown because SPI is a glycoprotein (Fig. 4) whose the molecular weight of carbohydrate has yet not been determined.

Most extracellular inhibitors interact with proteases, essentially irreversively with Ki ranging from 10^{-9} to 10^{-11} (Kassell and Williams, 1976). Carney *et al.* (1980) have proposed that in diluted extracellular situation it is fit for an inhibitor to inactivate its target protease irreversibly so that the reaction has to be rapid and complete. However, for an intracellular inhibitor, it would be much more useful to modulate an intracellular protease reversively (e.g. competitively) so that the protease could be switched on and off in response to cellular metabolic demands. SPI may work similarly to regulate protein catabolism in muscle cells.

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계 골격근에서 순수분리한 Serine Protease Inhibitor의 특성과 작용기구

김옥매 · 성성수 · 박혜경 · 최준호* · 성진하 · 하두봉 (서울대학교 자연대 동물학과, *한국과학기술원 생물공학과)

Trypsin을 비롯한 여러 serine protease를 저해하는 단백질을 계골격근으로부터 여러 chromatography 방법을 이용하여 순수하게 분리하였다. 이 저해제의 분자량을 젤 여과법을 이용하여 측정한 결과 66,000 달톤이었으며 sodium dodecyl sulfate 존재하에서 전기영동하였을 때 66,000과 64,000 달톤의 두 단백질로서 나타났다. 이 중 64,000 달톤의 단백질은 순수분리과정 혹은 생체 내에서 일어난 부분 절제현상에 기인한 66,000 달톤 단백질의 부산물인 것으로 사료된다. 이 저해제는 약 7.4의 등전점을 가진 당 단백질임을 알 수 있었으며, 다량의 cysteine잔기를 포함하고 있다.