BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **247,** 136–141 (1998)
ARTICLE NO. RC988747

Characterization of Trypsin-Modified Bovine Lens Acylpeptide Hydrolase

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Received April 23, 1998

Acylpeptide hydrolase, which removes the N-acetylated amino acids from peptide substrates was purified from bovine lens, truncated *in vitro* to a 55 kDa enzyme by trypsin digestion and characterized. The activity of the trypsin-modified enzyme was investigated using αA-crystallin and oxidized insulin A chain. The trypsin-modified enzyme was able to unblock αA-crystallin and displayed endoprotease activity unlike the native enzyme. SDS-PAGE analysis and amino acid sequencing of (^{3}H)iPr$_2$*P*-F labeled bovine lens acylpeptide hydrolase showed that the lens has a 55 kDa truncated form of the enzyme. The *in vivo* truncated form of the enzyme was generated by the cleavage of the Gly203-Asp204 peptide bond in the native enzyme.

Acylpeptide hydrolase (APH) belongs to a new class of serine-type peptidases containing a catalytic triad involving Ser, His and the β-carboxyl group of an aspartic acid residue (1, 2). It has been isolated from rat, bovine and porcine livers (3-6), rat brain (7), sheep erythrocyte (8), human erythrocyte (9, 10), rabbit muscle (11), human placenta (12), porcine and rat intestine (13, 14), and bovine lenses (15). The enzyme preferentially releases Ac-Ala, Ac-Ser and Ac- Met (16), the most common acetylated NH_2-terminal residues from peptide substrates but not from protein substrates (17, 18). It has been shown that if the proteins are first hydrolyzed by proteases, acylpeptide hydrolase is able to deblock the N-terminal peptide (18). The enzyme is inhibited by diisopropylfluorophosphate (iPr$_2$*P*-F), phenylmethylsulfonyl fluoride and N-ethyl maleimide, indicating the presence of an essential serine residue and -SH group (1-3, 10, 15, 20). Under denaturing conditions the native 300 kDa protein dissociates into 75 kDa subunits, each having one active site (2, 3, 15).

Recently, we described acylpeptide hydrolase isolated from bovine lens and showed that its properties were similar to the enzyme isolated from other sources with respect to the molecular weight and substrate specificity (15, 19). Digestion of the native APH with bovine trypsin generated a 55 kDa fragment containing the active site and a smaller 22 kDa fragment (15). The trypsin modified acylpeptide hydrolase (55 kDa fragment) sequence at the trypsin cleavage site was identical to the porcine liver acylpeptide hydrolase sequence 196-215 (15, 21). The native enzyme displayed remarkable resistance to urea denaturation (15). Subsequently we have also shown that the lens APH is active under in vivo conditions by assaying α-malanocyte stimulating hormone (22).

It has been shown that several amino acids from the N-terminal region of α-crystallin are removed during aging (23). The N-terminal truncation of β-crystallins during aging and cataract formation has also been documented (24). However the proteases responsible for the initial deacetylation or the removal of the N-acetylated amino acids from these proteins to allow the aminopeptidases to cleave additional amino acids from the N-terminus of the crystallins are not understood. In this study we investigated the properties of the 55 kDa form of APH prepared by trypsin digestion of the native enzyme. During the study we also observed that protease activity is associated with the in vitro truncated enzyme. We also provide evidence for the in vivo truncation of the enzyme in bovine lens.

MATERIALS AND METHODS

Materials. Bovine lenses were obtained from a local slaughterhouse and stored at −70°C until use. The substrates, N-acetyl-Ala-

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Abbreviations: iPr$_2$*P*-F, diisopropylfluorophosphate; APH, acylpeptide hydrolase; p-NA, p-nitroanilide; TLCK, N-tosyl-lysylchloromethylketone.

0006-291X/98 $25.00

p-nitroanilide (NA), N-formyl-Met-p-nitroanilide, N-acetyl-Phe-p-nitroanilide, N-acetyl-Leu-p-nitroanilide, L-alanyl-L-ala-p-nitroanilide and oxidized insulin chain A and the inhibitors, iPr_2P-F, soybean trypsin inhibitor and N-tosyl-lysylchloro-methyl ketone (TLCK) were obtained from Sigma Chemical Co. Trypsin (3× crystallized and treated with L- (tosylamido-2-phenyl)ethyl chloromethyl ketone) was bought from Worthington (Freehold, N.J.). (^{3}H)iPr_2P-F (specific activity, 8.4 Ci/mmole) was purchased from New England Nuclear. All other chemicals were the highest grade commercially available. HPLC column (218TP54, Vydac C_{18}, 10 mm × 250 mm) was supplied by The separations Group (Hesperia, CA, USA).

Acylpeptide hydrolase purification. Acylpeptide hydrolase was purified after a modification of the method of Sharma and Ortwerth (15). In brief, 30 de-capsulated bovine lenses were stirred with 50 mM Tris-HCL (buffer A, 5 ml per lens), pH 7.5 for 1 h. All procedures were performed at 4° C unless otherwise described. The soluble proteins from the disrupted lens fiber cells were decanted to separate the nuclei and centrifuged at 30,000×g for 30 min. The supernatant was collected and designated as the cortical crude lens extract. The crude lens extract protein was 60% saturated with solid $(NH_4)_2SO_4$. The precipitated proteins were separated by centrifugation at 10,000×g for 30 min. The pellet was re-suspended in 50 mM Tris-HCl, pH 7.5 (buffer A) and dialyzed overnight against the same buffer, with several buffer changes. The dialyzed solution was placed on a DEAE-Sephadex column (3×15 cm) previously equilibrated in buffer A. The column was washed extensively with buffer A containing 0.2 M NaCl. The bound proteins were eluted with a 400 ml linear gradient of increasing NaCl concentration (from 0.2 to 0.4 M in buffer A). Ten ml fractions were collected at a flow rate of 30 ml/h during the gradient elution. The active fractions were pooled and dialyzed against buffer A and concentrated to 7 ml by PD10 ultrafiltration membrane (Amicon Corp). The concentrated enzyme solution was subjected to gel filtration on a Sephadex G-200 column (2.5 × 70 cm) previously equilibrated with buffer A. Ten ml fractions were collected at a flow rate of 20 ml/h. Fractions possessing the enzyme activity were pooled and concentrated by ultrafiltration. The homogeneity and molecular mass of the purified enzyme was determined by SDS-PAGE. The purified enzyme was stored in 10% glycerol and 1 mM DTT at −20°C.

In a separate experiment, the partially purified APH fraction from lens cortical and nuclear fibers was subjected to SDS-PAGE under reducing conditions, transferred to PVDF membrane (25), and the protein band at 55 kDa region was excised. The excised protein band was later subjected to automated Edman degradation.

Acylpeptide hydrolase assay. Lens acylpeptide hydrolase activity was measured by using a synthetic substrate Ac-Ala-p-nitroanilide as described (15). Purified enzyme sample (10-100μl) was mixed with 50 mM Tris-HCl, pH 7.5 in a total volume of 0.99 ml. The reaction was initiated by the addition of 1 μmol Ac-Ala-p-nitroanilide dissolved in dimethylsulfoxide. The p-nitroaniline released was monitored in a Perkin Elmer Lambda 3 spectrophotometer at 405 nm. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 μmole of substrate per minute under the assay conditions.

Preparation of trypsin-modified acylpeptide hydrolase. Acylpeptide hydrolase was treated with TPCK treated bovine trypsin (10:1 ratio) in 1 ml 50 mM Tris-HCl, pH 7.5 and incubated at 25° C. After 30 min incubation, the trypsin activity in the reaction mixture was inhibited by the addition of an excess of soybean trypsin inhibitor. The trypsin treated sample was subjected to gel filtration on a Sephadex G-200 column (2.5× 70 cm) equilibrated with buffer A. Ten ml fractions were collected with the flow rate of 20 ml/h. Fractions showing APH activity were pooled and concentrated. The purity of the trypsin-modified enzyme was determined by 10% SDS-PAGE.

Labeling of the cortical and nuclear acylpeptide hydrolase as well as trypsin-modified acylpeptide hydrolase with (^{3}H)iPr_2P-F. Partially purified APH from both cortical and nuclear lens fibers as well as the trypsin modified enzymes were labeled with (^{3}H)iPr_2P-F as described earlier (15). Aliquots of the labeled enzyme were subjected to SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue R-250. The gels were destained and prepared for fluorography as described by Bonner and Laskey (26). Kodak X-Omat AR film was used to locate the labeled protein bands.

Hydrolysis of αA-crystallin by trypsin modified APH. Fifty μg of HPLC purified αA-crystallin (27) was incubated with 5 μg native APH or trypsin-modified APH in 50 mM Tris-HCl, pH 7.5 at 37° C for 18 h. After the incubations the samples were subjected to 15% SDS-PAGE and transferred to PVDF membranes (25). The protein band corresponding to αA-crystallin was excised and sequenced by automated Edman degradation.

Hydrolysis of oxidized insulin A chain by trypsin-modified 55 kDa APH. Oxidized insulin A chain (250 μg) was incubated with 25 μg of trypsin-modified APH in buffer A at 25° C. After 12 hrs of incubation, the reaction mixture was filtered through 10 K cut-off centrifuge filter (MSI, Westborough, MA). The filtrate, containing a mixture of hydrolyzed peptides, was injected into a Vydac C_{18} column (218TP54, 10 × 250 mm). The peptides were eluted with a 60 min linear gradient (0-60%) formed between water and acetonitrile containing 0.1% (W/V) trifluoro acetic acid. The absorbance was monitored at 220 nm during the elution period. All peptide peaks were collected separately and analyzed by sequencing to determine their identity.

Other methods. Protein/peptide sequencing was carried out in an Applied Biosystems 470A gas-phase protein sequencer coupled to an online phenylthiohydantoin-Xaa microbore analyzer. The protein concentration was determined by the bicinchonic acid method (28) using bovine serum albumin as a standard for calculation of specific activity. The stability and activity of the native and the trypsin-modified APH in urea concentrations 0-6.0 M was determined after 1 h exposure of the enzymes to varying urea concentrations using Ac-Ala-p-nitroanilide.

TABLE 1

Purification of Acylpeptide Hydrolase from Bovine Lens Extract

Fraction	Protein (A280)	Total activity (Units)	Specific activity (Units/A280)	Purification (-fold)	Yield (%)
Crude extract	12,590	2741	0.22	1	100
$(NH_4)_2SO_4$	14,482	2152	0.73	3	79
DEAE-Sephadex	4.2	1607	383	1741	59
Sephadex G-200	0.9	1341	1490	6772	49

The isolation procedure was started with 30 bovine lenses. Activity was determined with the substrate Ac-Ala-p-nitroanilide at 25° C and pH 7.5.

TABLE 2

Relative Activities of Native and Trypsin-Modified APH Towards Various Substrates

Substrates	Native APH	Trypsin-modified APH
Ac-Ala-pNA	100	100
Ac-Phe-Ala-pNA	0	0
Ac-Leu-pNA	0	0
L-Ala-L-Ala-pNA	1	1.8
Formyl-Met-pNA	66	78.6

The relative rate of hydrolysis was determined as described under methods using 1.0 mM concentration of each substrate except N-formyl-Met-pNA which was used at 0.25 mM due to its lower solubility.

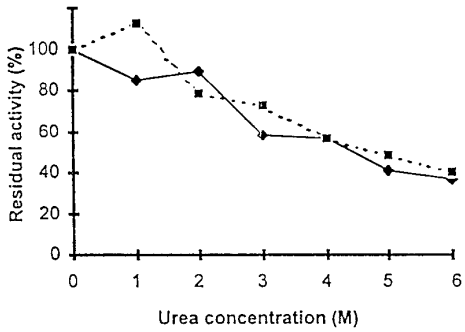

FIG. 1. Effect of urea on native and trypsin-modified APH activity. Aliquots of native and trypsin-modified APH were mixed with 0-6 M urea in buffer A. After 1 h at room temperature, the residual activity was measured using Ac-Ala-p-nitroanilide. ♦, native APH; ■, trypsin-modified APH.

RESULTS

Acylpeptide hydrolase purification. Acylpeptide hydrolase (APH) was purified from bovine lens extract by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography and gel filtration. The summary of the purification is shown in Table 1. A three step purification procedure resulted in over 6,000 fold purification of the enzyme with 49 percent recovery of the activity. The homogeneity of the purified enzyme was determined by SDS-PAGE. A single protein band of 75 kDa was observed under dissociating conditions (Figure not shown).

Trypsin-modified APH and its properties. Earlier we reported that digestion of lens APH with trypsin generates a 55 kDa form of the enzyme and a N-terminal 22 kDa fragment (15). During the present study, trypsin-modified APH was prepared as described earlier and the activity of the 55 kDa fragment towards synthetic and protein substrates was determined. The activity of the trypsin-modified APH and the native APH towards different substrates is shown in Table 2. While both enzymes showed similar activity when Ac-Ala-p-nitroanilide was used as a substrate, the trypsin modified enzyme showed marginally higher activity towards N-formyl-Met-p-nitroanilide. The Km and V max for the native enzyme were 0.4 ±0.03 mM and 680 ± 50 nmole/min/mg protein respectively. These values were comparable to the Km and Vmax of 0.27 ± 0.09 mM and 890 ± 22 nmole/min/mg for the trypsin-modified enzyme. The pH optimum of the trypsin-modified enzyme was found to be in the range 7.5-8.0 when tested with Ac-Ala-p-nitroanilide as a substrate. The effect of varying concentrations of urea on native and trypsin modified enzyme activity is shown in Fig. 1. Both enzymes showed decreased activity with increasing urea concentration. In the presence of 6 M urea, both enzymes showed 30 percent of their original activity under our experimental conditions.

The ability of trypsin-modified APH to unblock acetylated protein was investigated using bovine lens αA-crystallin. αA-crystallin was digested with the trypsin-modified APH and subjected to SDS-PAGE under reducing conditions and transferred to PVDF membrane. The sequencing of 20 kDa protein band by Edman degradation method gave an N-terminal sequence DIAIQH for the protein. This sequence corresponds to the residues 2-7 in bovine lens α-crystallin (29). Fig. 2 shows the SDS-PAGE of α-crystallin digested with either native APH or the trypsin-modified APH. The data shows that the trypsin-modified APH can cleave αA-crystallin and generate a ~19 kDa protein (lanes 6 and 7 in Fig. 2). The cleavage of αA-crystallin was seen in the presence of added TLCK. However, addition of iPr_2P-F completely abolished this activity (lane 8, Fig. 2). The na-

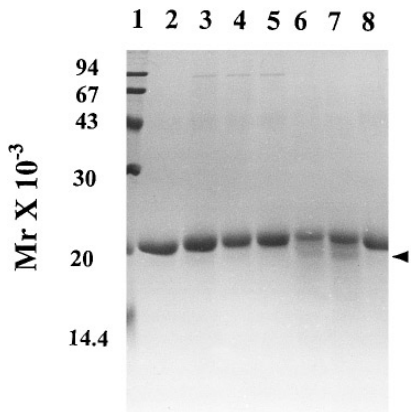

FIG. 2. SDS-PAGE of αA-crystallin digested with APH. αA-crystallin was digested with either native or trypsin modified APH as described under methods and subjected to SDS-PAGE. Lane, 1, molecular weight standards; lane 2, αA-crystallin; lane 3, αA-crystallin + native APH; lane 4, αA-crystallin + native APH + TLCK; lane 5, αA-crystallin + native APH + TLCK + iPr_2P-F; lane 6, αA-crystallin + trypsin-modified APH; lane 7, αA-crystallin + trypsin-modified APH + TLCK; lane 8, αA-crystallin + trypsin-modified APH + TLCK + iPr_2P-F.

TABLE 3

Trypsin-Modified Acylpeptide Hydrolase Cleavage Sites in Oxidized Insulin A Chain

Gly-Ile-Val-Glu-Gln-Cys(SO_3H)-Cys(SO_3H)Ala↓Ser-Val-Cys(SO_3H)-Ser-Leu-Tyr↓Gln-Leu-Glu-Asn-Tyr Cys(SO_3H)-Asn

Insulin A chain was incubated with trypsin-modified APH for 12 hrs and the reaction product was analyzed by HPLC. The peptide peaks were subjected to amino acid sequencing as described under methods. The arrow mark indicated 12 h cleavage site.

tive APH under similar conditions was unable to cleave αA-crystallin (lanes 3-5 in Fig. 2). We also observed the cleavage of purified αB-crystallin by trypsin-modified APH in a separate experiment (data not shown).

We used oxidized insulin A chain as a substrate to confirm the endoprotease activity of the trypsin-modified APH. Trypsin-modified APH cleavage sites in insulin A chain after 12 hr digestion is shown in Table 3. These cleavages were abolished by prior inactivation of trypsin-modified APH with iPr_2P-F.

Bovine lens has truncated form of APH. Recent studies have shown that the N-terminus of both αA- and αB- crystallins is truncated in vivo (23). However, the proteases responsible for this modification have not been identified so far. In view of the fact that the in vitro generated, truncated form of APH was able to cleave αA-crystallin, we investigated whether the lens has any truncated form of APH. Partially purified APH from bovine lens cortical and nuclear fibers was labeled with (^{3}H)iPr_2P-F and subjected to SDS-PAGE and fluorography. The results are shown in Fig. 3. Both cortical and nuclear preparations showed four (^{3}H)iPr_2P-F labeled protein bands in the 30-75 kDa region (lanes 6 and 7). The 75 kDa band represents the labeled subunit of intact APH (15). The additional three bands represent either a truncated form of APH or separate serine proteases. Fig. 3 also shows the SDS-PAGE profile of the trypsin-modified APH in vitro (lanes 4 and 7). Both the cortical and nuclear preparations showed a 55 kDa labeled protein, similar to the trypsin-modified APH (compare lanes 5 and 6 with 7 in Fig. 3). The 55 kDa protein band from the cortical preparation was excised and subjected to Edman degradation to determine the identity of the labeled protein. The N-terminal amino acids identified by amino acid sequencing, DQFLFYE, correspond to the amino acid residues 204-210 in APH isolated from bovine lens (15), porcine liver (21) and rat liver (3). The results indicate that APH was cleaved at the peptide bond between Gly203-Asp204 to generate the 55 kDa form of APH in vivo. Earlier we reported that the trypsin-modified APH has the N-terminal sequence KPDQAIKGDQFLFYED(X)GEN (15), identical to the pig liver enzyme sequence 196-215 (21). Since the ~65 kDa protein band was not sufficiently pure and there was insufficient material in the ~30 kDa protein region, the identity of ~65 kDa and ~30 kDa (^{3}H)iPr_2P-F labeled proteins in either cortical or nuclear preparations is not known at present.

DISCUSSION

Bovine lens acylpeptide hydrolase, purified over 6700 fold by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography and gel-filtration, revealed a 75 kDa subunit under denaturing SDS-PAGE as previously reported (15). Digestion of the native enzyme with trypsin resulted in the generation of a 55 kDa subunit containing enzyme with about a 12 percent loss in activity against the synthetic substrate Ac-Ala-p-nitroanilide. The activity of the trypsin-modified enzyme toward formyl-Met-p-nitroanilide was marginally higher than that of the native enzyme (Table 2). The relative activities of the native as well as the trypsin-modified enzymes toward N-acetylated substrates were higher than toward N-formylated substrates. The pH optimum, Km and Vmax of the trypsin-modified APH were similar to that of the native enzyme. These values are comparable to those reported for the APH isolated from other sources.

A significant difference between the trypsin-modified APH and the native APH was discovered when both enzymes were tested for their ability to hydrolyze αA-crystallin and oxidized insulin A chain. The trypsin modified APH was able to cleave off Ac-Met from the N-terminus of αA-crystallin and expose Asp2 to allow sequencing of the protein. In addition, we also observed cleavage of αA-crystallin (Fig. 2) by the trypsin-modi-

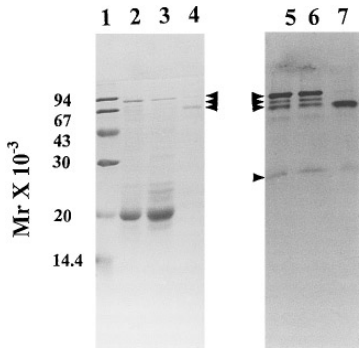

FIG. 3. Comparison of SDS-PAGE pattern of partially purified and trypsin-modified APH labeled with (^{3}H)iPr_2P-F. APH was partially purified from bovine lens cortical and nuclear fibers. An aliquot of this preparation was labeled with (^{3}H)iPr_2P-F as described under methods. Simultaneously, 5 μg of the trypsin-modified APH was also labeled with (^{3}H)iPr_2P-F. An aliquot of each sample was subjected to SDS-PAGE, stained, treated with 2,5-bis(2-(4-methyl-5-phenyloxazolyl))-benzene and exposed to X-ray film. Lanes 1-4, Coommassie stain. Lanes 5-7, Fluorography. Lane 1, molecular weight standards; lanes 2 and 5, APH from lens cortical fibers; lane 3 and 6, APH from lens nuclear fibers; lanes 4 and 7, trypsin-modified APH.

fied APH, which could be inhibited by iPr_2P-F. Furthermore, trypsin-modified APH was able to cleave oxidized insulin A chain (Table 3), demonstrating endopeptidase activity. The native APH under similar conditions was unable to cleave αA-crystallin or insulin A chain. On the basis of these data, we hypothesize that while the removal of ~22 kDa N-terminal fragment from APH by trypsin cleavage makes the enzymes active site more accessible for larger protein substrates; in the native enzyme, the ~22 kDa N-terminal portion offers steric hindrance to protein substrates. The peptide substrates easily diffuse to the active site in the native enzyme and get cleaved as in the case of trypsin bound to α_2-macroglobulin, which can hydrolyze chromogenic substrates such as N-benzoyl-Arg-p-nitroanilide and not casein (29). This can be confirmed once the crystal structure for native and trypsin-modified APH becomes available.

In view of the ability of the trypsin-modified 55 kDa APH to cleave α-crystallin, we hypothesized that if a similar truncation of APH occurs in vivo and the resultant modified APH cleaves α-crystallin, the in vivo truncation of α-crystallin reported recently by Kamei et al (23) can be explained. We were able to show the in vivo truncation of bovine lens APH by (^{3}H)iPr_2P-F labeling, and amino acid sequencing studies. When we labeled the partially purified APH from lens with (^{3}H)iPr_2P-F, a 55 kDa protein with the same mobility as the trypsin-modified APH was seen after SDS-PAGE (Fig. 3). Amino acid sequencing confirmed that it was an in vivo truncated form of the APH generated by cleavage of Gly203-Asp204 bond. This cleavage site is near the unique trypsin cleavage site, between Lys195 and Lys196 in bovine APH we reported earlier (15). The protease responsible for the in vivo cleavage of APH is not known at present. However, it is unlikely that the 55 kDa APH was generated in vivo by differential splicing since alternate splice sites in APH gene have not been reported so far. Preliminary studies with human lens APH preparations also indicate the presence of a 55 kDa truncated form of APH. Further studies are underway to determine the in vivo cleavage sites in human lens APH.

Since it has been suggested that the N-terminal region of α-crystallin may also play an important role in chaperone-like activity of the protein (31, 32), it is conceivable that the truncated form of APH through its action on α-crystallin can play a direct role in modulating the function of α-crystallin. Additionally, earlier studies have shown that N-terminal region of α-crystallin may be involved in binding of the protein to the membranes (33). This binding process has been implicated in cataractogenesis. These data suggest that proteolytic enzymes can play a pivotal role in cataractogenesis in different ways.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Research to Prevent Blindness, Inc. and in part by the National Institute of Health grant EY 09855. The authors thank Dr. B. J. Ortwerth for helpful discussion.

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