BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 365–370 (1996)
Article No. 0064

Identification of New Lens Protease(s) Using Peptide Substrates Having *in Vivo* Cleavage Sites

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Received November 29, 1995

Bovine lens extracts were incubated in pH 7.5 buffer with succinylated VSREEKPSSAPSS, SGVDAGHS, GKPTSAPSS and GKHNERQD, the peptides having age-dependent in vivo cleavage sites in bovine and human αA-crystallin. The reaction products were analyzed to identify the initial protease cleavage sites. The results showed that bovine lens extracts contain protease activity that can cleave. Thr-Ser, Ser-Ala, Ser-Ser bonds in test substrates which correspond to peptide bonds Ser168-Ser169, Ser169-Ala170, Ser172-Ser173 in bovine αA-crystallin and Thr-Ser, Ser169-Ala170, Ser172-Ser173 in human αA-crystallin. In addition, the same extracts were also capable of hydrolyzing Asp-Ala and Asn-Glu bonds corresponding to Asp151-Ala152 and Asn101-Glu102 in αA-crystallin from bovine and human lenses. The cleavage specificity of the newly discovered protease(s) suggests that the in vivo truncation of αA-crystallin reported earlier may be due to the action of proteases. The newly discovered lens proteases(s) were resistant to inactivation by E-64 and DFP. However, prior treatment of the lens extracts with leupeptin and chymostatin resulted in partial loss of Asn-Glu hydrolytic activity. N-ethylmaleimide treatment completely abolished in the Asn-Glu hydrolyzing activity.

The structural proteins of the lens (crystallins) are largely present throughout the life of the organism. Alpha crystallin is the major component of crystallin proteins accounting for as much as 40% of the total protein in lens. It is composed of two types of subunits, A and B of 20 kDa with extensive sequence homology to small heat shock proteins (1, for a review). Recently it has been shown that both A and B subunits have the capacity to suppress the aggregation and precipitation of denatured proteins (2). In vitro studies have demonstrated that the α-crystallin isolated from older lenses (3) as well as chemically modified (4,5) and enzymatically truncated (6,7) α-crystallins have diminished capacity to protect denatured β- and γ-crystallins and other proteins from insolubilization. Takemoto et al. have shown that the C-terminal region of α-crystallin is essential for the normal interaction of crystallin subunits as well as for its full chaperone-like function (6,8,9). Based on these observations it has been hypothesized that α-crystallin may be involved in the maintenance of lens transparency in vivo (2,3). During aging and cataract development, truncation of α-crystallin due to extensive cleavages at the carboxyl terminus has been observed by a number of investigators (10–14). Therefore it is likely that the loss in the chaperone-like property of aged α-crystallin observed (3) may be in part due to the C-terminal truncation of α-crystallin.

By isoelectric focusing, peptide mapping and amino acid analyses, van Kleef and associates (10,11) have determined that major bonds cleaved during aging in vivo are on the carboxyl side of Asn-101, Asp151, Ser168, Ser169 of the A chain and Thr170 of the B chain of bovine α-crystallin. Takemoto has shown that aged bovine αA-crystallin is cleaved in vivo on the carboxyl side of Ser168 and Ser172 (14). His study also showed the cleavage of human αA-crystallin on the carboxyl side of Thr-168 and Ser172 (14). Smith et. al. reported and confirmed independently some of these cleavage sites in α-crystallin (13,15). Of the above cleavages in bovine α-crystallin, only the cleavage site of αB-crystallin by calpain (16). Although the lens is known to contain a number

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Abbreviations used: E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; HPLC, high performance liquid chromatography; DFP, diisopropyl fluorophosphate.

0006-291X/96 $12.00

of other proteases (17–21), specific proteases responsible for in vivo truncation of α-crystallins have not been characterized. In vitro studies have indicated that non-enzymatic mechanisms may be playing a role in the truncation of crystallins in vivo (11,22,23). We hypothesize that lens tissue contains specific protease(s) which recognize certain sequences and they can be assayed only with peptide/protein substrates having sequences similar to that of a crystallin. Earlier studies have demonstrated that the peptide substrates having the in vivo cleavage sites are useful in identifying the sequence specific proteases, developing of rapid assays and designing specific inhibitors of therapeutic importance (24–26). In this study we provide evidence for the presence of proteases in lens which have a potential to cleave peptide bonds corresponding to the age related in vivo cleavage sites in α-crystallin. The data presented here were obtained using synthetic peptide substrates having sequences similar to the α-crystallin at the in vivo cleavage sites.

MATERIALS AND METHODS

Materials. Peptide substrates Suc-VSREEKPSSAPSS, Suc-SGVDAGHS, Suc-GKPTSAPSS and Suc-GKHNERQD were synthesized at the Protein Core Facility of Missouri, Columbia. Porcine kidney cytosolic leucine aminopeptidase, DFP, E-64, bestatin, chymostatin, leupeptin and N-ethylmeleimide were obtained from Sigma Chemical Co. HPLC column, Vydac C18, was bought from The Separations Group (Hesparia, CA, U.S.A). Bovine lenses were obtained from a local slaughter house and stored at −70°C until use. Human lenses were collected from eyes donated to the Lion's Eye Tissue Bank of Missouri and stored at −20°C. All other chemicals were the highest grade commercially available.

Methods. Bovine (–5 yrs) and human (4–70 yrs) lenses were homogenized in 50 mM phosphate buffer, pH 7.5 (buffer A) and the soluble proteins were separated by centrifugation at 30000Xg for 30 min. The extracts were dialyzed against 100 volumes of the same buffer for 12 hrs with one change using a 10 kDa cut off cellulose tubing.

Assay. In a typical assay, 20–50 nanomoles of the test peptide was mixed with ∼10 mg lens protein in buffer A under sterile conditions for –24 hrs. The total reaction mixture was then filtered through a 10 kDa cutoff filter (MSI) and the filtrate was subjected to HPLC analysis using a Vydac C18 column (4.5 × 250 mm). A linear gradient of 0.1% TFA in water and acetonitrile containing 0.1% TFA was used to separate the peptides generated by proteolysis. The peptide peaks were collected separately and analyzed for amino acid composition (27). In some experiments DFP, E-64, leupeptin, chymostatin, N-ethylmaleimide or bestatin was included to inactivate the proteases and peptidases known to be present in lens extracts prior to incubations with peptide substrates. The extent of hydrolysis was determined either by HPLC analysis of the 10 kDa filtrates or by amino acid analysis of the reaction products. Appropriate blanks were run under identical conditions and corrected for any hydrolysis of endogenous substrates. Protein was measured by the bicinchoninic acid method (28).

RESULTS AND DISCUSSION

The work described in this paper explores the use of peptide substrates representing in vivo cleavage sites in α-crystallins (1,10–15,29) for the identification of lens proteases. The synthetic peptides (Fig. 1) used in this study were all blocked at the N-terminus by succinylation to prevent their hydrolysis by the aminopeptidases present in lens (30–34).

Hydrolysis of human lens αA-crystallin peptides by bovine lens proteases. Figure 2 shows the

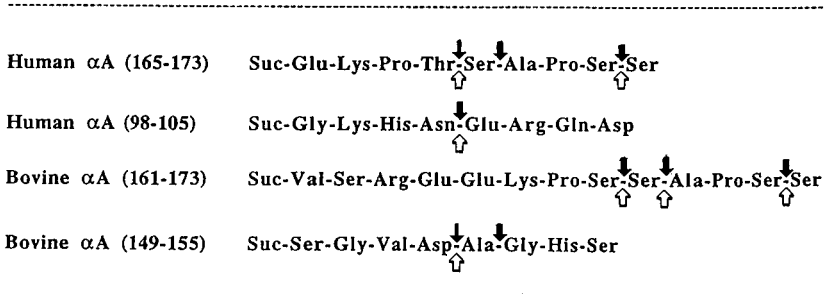

FIG. 1. Comparison of in vivo cleavage sites of αA-crystallin from bovine and human lens with that determined by in vitro studies. The in vivo cleavage sites (unfilled arrows) shown were taken from ref. 10–14 for bovine αA-crystallin and ref. 12, 14, 15 for human αA-crystallin. The filled arrows show the bovine lens protease cleavage sites observed during the present study. The assay method is described under methods.

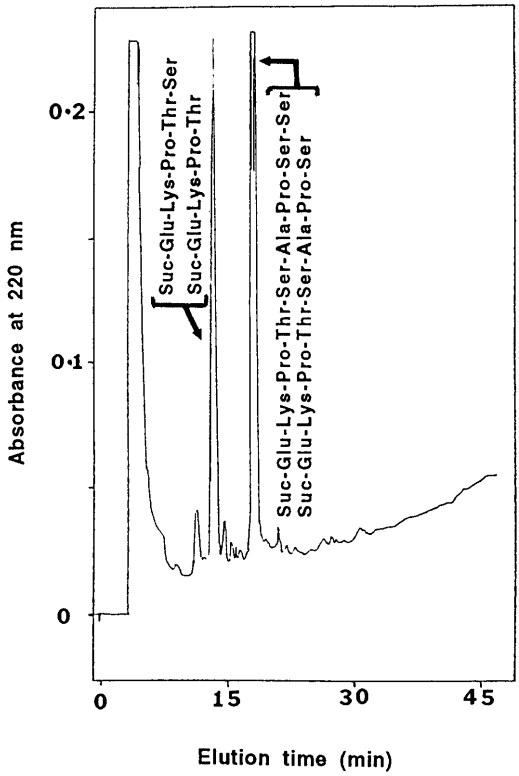

FIG. 2. Elution profile of Suc-GKPTSAPSS incubated with bovine lens extract during HPLC on a reverse phase C18 column. The experimental detail is given under methods.

HPLC profile of the peptide substrate, Suc-GKPTSAPSS incubated with the bovine lens extract. During chromatography on a C18 column, the original substrate eluted at 18 min with a water/ acetonitrile gradient. The same substrate, after incubation with 10 mg of DFP treated lens protein for 12 hrs, showed an additional peak at 14 min during HPLC separation of the products. The amino acid analysis of this new peak indicated that it was Suc-GKPTS suggesting the cleavage at Ser-Ala bond in the substrate used. The amino acid analysis of the substrate peak showed 10% decrease in total serine. This was due to the co-elution of Suc-GKPTSAPSS and Suc-GKPTSAPS. In addition, direct amino acid analysis of the reaction products showed the presence of free Ala, Pro and Ser. When the substrate was incubated with 10 times more bovine lens enzyme, 100% cleavage of Ser-Ala bond was observed. In addition, the amino acid composition analysis of the 14 min peak (Suc-GKPTS) showed a 15% decrease in Ser suggesting additional cleavage at Thr-Ser bond. The Thr-Ser bond was less susceptible to bovine lens protease compared to Ser-Ala bond. These results show the presence of protease(s) in bovine lens extract that cleaves Thr-Ser, Ser-Ala and Ser-Ser bonds in test substrates (Fig. 1). Of these three sites, Thr-Ser and Ser-Ser cleavage sites correspond to the in vivo truncation sites in human αA-crystallin (14,15).

In another set of experiments, the peptide substrate, Suc-GKHNERQD, corresponding to human αA-crystallin residues 98–105, containing in vivo cleavage site Asn101-Glu102 was incubated with the bovine lens extract. Amino acid analysis of the products showed the presence of free Glu, Arg, Gln and Asp that increased in a time dependent manner (Table 1). Incubation of the peptide alone under similar conditions did not show the generation of new amino terminus as judged by TLC and ninhydrin staining (data not shown). In addition, incubations of the peptide substrate for 24 hrs with purified leucine aminopeptidase did not result in the release of any one of the amino

TABLE 1
Amino Acids Released from Synthetic Peptide Substrates by Lens Protease(s)

Peptide	Incubation time	Amino acid released (nanomoles)
Bovine αA (148–155) (Suc-SGVDAGHS)	14 hrs	Ala(2.1); Gly(13); His(12); Ser(13)
Human αA (98–105) (Suc-GKHNERQD)	1 hr	Glu(3.5); Arg(2.3); Gln(1.1); Asp(0.6)
	2 hrs	Glu(4.6); Arg(3.5); Gln(1.2); Asp(1.0)
	4 hrs	Glu(6.1); Arg(4.6); Gln(2.4); Asp(2.1)
	6 hrs	Glu(8.9); Arg(8); Gln(3.8); Asp(3.6)
Human αA (98–105) (Suc-GKHNERQD) + Leucine aminopeptidase	24 hrs	None

The peptide substrates (25 nanomoles) were incubated with dialyzed bovine lens extract or 50 μg of purified leucine aminopeptidase for 1–24 hrs and the products were analyzed for free amino acids as described under methods. The values shown represent amino acids released after correcting for any endogenous amino acid released.

acids present in the peptide. If there was any non-enzymatic cleavage of the substrate Suc-GKHNERQD, we should have seen the release of free amino acids from the newly formed peptides by the action of leucine aminopeptidase. Therefore, cleavage of the Asn-X bond in the peptide substrate was unlikely to be due to a non-enzymatic cleavage occurring under our experimental conditions. Non-enzymatic cleavage of Asn-X bonds in peptides and proteins during prolonged incubation under different conditions has been observed by others (22,35). The data from this study suggests that the peptide we used was initially cleaved at Asn-Glu bond and that the lens peptidases hydrolyzed the peptide EQRD to release Glu, Arg, Gln and Asp. The results from this experiment suggest that the bovine lens extract contains a protease capable of hydrolyzing the Asn-Glu bond in the substrate used. Both human and bovine lens αA-crystallins are cleaved in vivo during aging at Asn101-Glu102 site, Fig. 1 (1,11).

Hydrolysis of bovine lens αA-crystallin peptides by bovine lens proteases. Bovine lens extract (10 mg protein treated with DFP to inactivate prolyloligopeptidase (36,37)) was incubated with 50μg of Suc-VSREEKPSSAPSS (corresponding to the succinylated bovine lens αA-crystallin sequence 161–173) for 16 hrs. The reaction product was analyzed by HPLC as above. The elution profile of the products showed several peptides (elution profile not shown). The individual peaks were collected separately and analyzed for amino acid content. The amino acid compositions of those peptides were equivalent to Suc-VSREEKPS, Suc-VSREEKPSS, and Suc-VSREEKPSSAPS. The Ser-Ser and Ser-Ala bonds were assigned as cleavage sites (Fig. 1) after comparison with the amino acid content of the starting peptide substrate. The analysis of the reaction products showed free Ser, Ala and Pro. The Ser-Ser and Ser-Ala cleavage sites in the substrate used correspond to the in vivo peptide bond cleavage sites Ser168-Ser169, Ser169-Ala170 and Ser172-Ser173 in bovine lens αA-crystallin (10,11,14).

In another experiment, DFP treated bovine lens extract (10 mg protein) was incubated with 50 μg of Suc-SGVDAGHS, corresponding to the succinylated bovine lens αA-crystallin sequence 148–155. The result obtained upon amino acid analysis of the reaction products is shown in Table 1. The data suggest that the peptide substrate was first cleaved at Asp-Ala and Ala-Gly bonds and the new peptides formed (AGHS and GHS) were hydrolyzed by lens aminopeptidases. Neither the presence of carboxypeptidase (30) nor the proteases discovered in the present study were detected in the lens so far. Our previous attempts to identify the presence of carboxypeptidase activity in lens extract were not successful. Therefore the free amino acids formed during our assay are unlikely to arise by the action of carboxypeptidase(s). Of the two cleavage sites identified during our in vitro

studies with Suc-SGVDAGHS, the Asp-Ala bond cleavage corresponds to Asp151-Ala152 bond in native bovine αA-crystallin, Fig. 1 (1,10,11).

Other properties of bovine lens proteases. The susceptibility of the newly discovered bovine lens protease(s) to various inhibitors was tested by in vitro assays with Suc-GKPTSAPSS and Suc-GKHNERQD as substrates. During the assays the Ser-Ala and Asn-Glu bond cleavages were monitored to determine the extent of inhibition. Both the activities were resistant to DFP and E-64. While a partial inhibition of the Asn-Glu hydrolyzing activity by leupeptin and chymostatin was observed, addition of N-ethylmaleimide to the assay system completely abolished the release of amino acids. These inhibitors by themselves do not inactivate the peptidases. Therefore the inhibition observed was likely due to inactivation of the protease acting on Asn-Glu site. These data suggest that the Asn-Glu hydrolyzing activity is similar to that of the cysteine proteases whereas the Ser-Ala hydrolyzing activity does not belong to any specific class of known proteases (38). To our best knowledge this is the first report of the presence of an Asn-X bond cleaving protease in mammalian tissue. The optimum pH of the lens protease(s) was 7.5. When the lens extract was passed through a Sephadex G-100 column the protease activities co-eluted with 70 kDa proteins under non-denaturing conditions. The molecular weight and inhibitory susceptibility of the bovine lens protease(s) discovered now are different from those reported earlier for calpain (19,20), multicatalytic protease (18), trypsin-like protease (17) and membrane protease (20) present in bovine lens extract.

The results from this study, for the first time, show that the bovine lens extract contains proteases with the potential to cleave αA-crystallins at specific sites corresponding to the age related in vivo cleavage sites. Similar activity was also observed in a limited number of human lens extracts analyzed (data not shown). In view of the earlier report that α-crystallin gets truncated during aging (10–14,39,40) and the chaperone-like function of α-crystallin is altered by this modification (6,9), it is possible that the protease(s) we identified may play a major role in the maintenance of α-crystallin structure-function in vivo. Further studies are underway to determine the role of these proteases in in vivo truncation of α-crystallin during aging and modulation of α-crystallin properties.

ACKNOWLEDGMENTS

The authors would like to thank Mr. P.R. Olesen for his help in the procurement of bovine lenses and Dr. B.J. Ortwerth for discussions and critical review of the manuscript. This work was supported by grants from NIH (EY 09855), University of Missouri Research Board and by an unrestricted grant from Research to Prevent Blindness Inc.

REFERENCES

1. Groenen, P. J. T. A., Merck, K. B., De Jong, W. W., and Bloemendal, H. (1994) *Eur. J. Biochem.* **225,** 1–19.
2. Horwitz, J. (1993) *Invest. Ophthalmol. Vis. Sci.* **34,** 10–22.
3. Horwitz, J., Emmons, T., and Takemoto, L. (1993) *Curr. Eye Res.* **11,** 817–822.
4. Sharma, K. K., and Ortwerth, B. J. (1995) *Exp. Eye Res.*
5. Cherian, M., and Abraham, E. C. (1995) *Biochem. Biophys. Res. Comm.* **208,** 675–679.
6. Takemoto, L., Emmons, T., and Horwitz, J. (1993) *Biochem. J.* **294,** 435–438.
7. Kelly, M. J., David, L. L., Iwasaki, N., Wright, J., and Shearer, T. R. (1993) *J. Biol. Chem.* **268,** 18844–18849.
8. Ifeanyi, F., and Takemoto, L. J. (1991) *Curr. Eye Res.* **10,** 529–535.
9. Takemoto, L. (1994) *Exp. Eye Res.* **59,** 239–242.
10. van-Kleef, F. S. M., de Jong, W. W., and Hoenders, H. J. (1975) *Nature* **258,** 264–266.
11. van-Kleef, F. S. M., Williems-Thijssen, W., and Hoenders, H. J. (1976) *Eur. J. Biochem.* **66,** 477–483.
12. Emmons, T., and Takemoto, L. (1992) *Exp. Eye Res.* **55,** 551–554.
13. Miesbauer, L., Zhou, X., Yang, Z., Sun, Y., Smith, D., and Smith, J. (1994) *J. Biol. Chem.* **269,** 12494–12502.
14. Takemoto, L. J. (1995) *Curr. Eye Res.* **14,** 837–841.
15. Yang, Z., Chamorro, M., Smith, D. L., and Smith, J. B. (1994) *Curr. Eye Res.* **13,** 415–421.
16. Yoshida, H., Yumato, N., Tsukahara, I., and Murachi, T. (1986) *Invest. Ophthalmol. Vis. Sci.* **27,** 1269–1273.
17. Tse, S., and Ortwerth, B. (1981) *Exp. Eye Res.* **32,** 605–614.
18. Wagner, B., Fu, J., Margolis, J., and Fleshman, K. (1984) *Exp. Eye Res.* **38,** 477–483.

19. Yoshida, H., Murachi, T., and Tsukahara, I. (1984) *Biochem. Biophys. Acta.* **798,** 252–259.
20. Srivastava, O. P. (1988) *Exp. Eye Res.* **46,** 269–283.
21. Shearer, T. R., David, L. L., Anderson, R. S., and Azuma, M. (1992) *Curr. Eye Res.* **11,** 357–369.
22. Voorter, C. E. M., Roersma, E. S., Bloemendal, H., and de Jong, W. W. (1987) *J. Biol. Chem.* **263,** 19020–19023.
23. Seizen, R. J., and Hoenders, H. J. (1979) *Eur. J. Biochem.* **96,** 431–440.
24. Kotler, M., Katz, R. A., Danho, W., Leis, J., and Skalka, A. M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4185–4189.
25. Kotler, M., Danho, W., Katz, R. A., Leis, J., and Skalka, A. M. (1989) *J. Biol. Chem.* **264,** 3428–3435.
26. Netzel-Arnet, S., Mallya, S. K., Nagase, H., Hanson, B. H., and Van Wart, H. E. (1991) *Anal. Biochem.* **195,** 86–92.
27. Gehrke, C. W., Rexroad, P. R., Schisla, R. M., Absheer, J. S., and Zumwalt, R. W. (1987) *J. Assoc. Anal. Chem.* **70,** 171–174.
28. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goenke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
29. Caspars, G.-J., Pennings, J., and de Jong, W. (1994) *Exp. Eye. Res.* **59,** 125–126.
30. van Heyningen, R., and Waley, S. G. (1963) *Biochem. J.* **86,** 92–100.
31. Swanson, A. A., Davis, R. M., Jackson, B. A., and McDonald, J. K. (1981) *Exp. Eye. Res.* **32,** 163–173.
32. Taylor, A., Daimes, A. M., Lee, J., and Surgenor, T. (1983) *Curr. Eye res.* **2,** 45–56.
33. Lafferty, M. A., Raducha, M., and Harris, H. (1984) *Curr. Eye Res* **3,** 1017–1031.
34. Sharma, K. K., and Ortwerth, B. J. (1986) *Curr. Eye Res.* **5,** 373–380.
35. Geiger, T., and Clarke, S. (1987) *J. Biol. Chem.* **262,** 785–794.
36. Swanson, A. A., Davis, R. M., and McDonald, J. K. (1984) *Curr. Eye Res.* **3,** 659–661.
37. Sharma, K. K., and Ortwerth, B. J. (1994) *Exp. Eye Res.* **59,** 107–116.
38. Barrett, A. J. (1995) *in* Methods in Enzymology (Barrett, A. J., Ed.), Vol. 244, pp. 1–15. Academic Press, London, UK.
39. Takemoto, L., and Gopalakrishna, S. (1994) *Curr. Eye Res.* **13,** 879–883.
40. Takemoto, L. (1995) *Exp. Eye Res.* **60,** 721–724.