Biochimica et Biophysica Acta, 327 (1973) 138–145

BBA 67057

A NEW RADIOACTIVE ASSAY FOR ENZYMES WITH ELASTOLYTIC ACTIVITY USING REDUCED TRITIATED ELASTIN. THE EFFECT OF SODIUM DODECYL SULFATE ON ELASTOLYSIS

SHIZUKO TAKAHASHI, SAM SEIFTER AND FRANCES CHANG YANG

The Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y. 10461 (U.S.A.)

(Received May 25th, 1973)

SUMMARY

A method is described for the assay of elastolytic activity based on the use of an insoluble elastin substrate the aldehydes and cross-links of which have been labeled by reduction with tritiated $NaBH_4$. The rate of appearance of radioactivity in the soluble phase of a digest appears to be a more sensitive and better index of elastolysis than both the rates of appearance of materials that react with ninhydrin or with the biuret–Folin reagent. Experiments were also performed to determine whether the reported enhancement of elastolysis occasioned by pretreatment of the substrate with sodium dodecyl sulfate could be verified using the radioactivity assay. Indeed the rate of elastolysis was approximately doubled when the labeled substrate was first treated with sodium dodecyl sulfate. Comparison of the rates of liberation to the soluble phase of radioactivity, ninhydrin-reacting material and of peptides reacting with the biuret–Folin reagent permits an inference that sodium dodecyl sulfate, in addition to inducing formation of new regions of α helix, may expose the polypeptide regions surrounding the cross-links allowing elastase subsequently to cleave these regions more rapidly.

INTRODUCTION

The usual substrate for measurement of elastolytic activity is one kind or another of insoluble fibrillar elastin. Actual elastolytic activity is then determined by the extent of solubilization of the elastin measured by an increase of ninhydrin reactivity reflecting peptide scission with appearance of amino groups, or an increase in biuret–Folin reagent reactivity reflecting appearance of soluble peptides[1]. Alternatively, one may measure release of fluorescent material reflecting the cleavage of polypeptide chains carrying as yet poorly characterized fluorescent material[2], or one may determine release of peptide-bound dyes such as Congo Red[3] or orcein[4] previously attached artificially to the insoluble elastin. Methods employing a pH-stat

have also been used (see, for instance, Robert and Robert[5]). Synthetic substrates for elastase have been described (see, for instance, Thompson and Blout[6,7]). Finally, a method recently has been described in which finely ground elastin is dispersed in a solid agar medium containing sodium dodecyl sulfate, and elastolytic activity determined by appearance of clear zones surrounding the site of application of the elastolytic enzyme (Faris, B., Kagan, H. M., and Franzblau, C., personal communication).

Because of the nature of the insoluble elastin substrate, assays based on its use have inherent problems related to difficulties of uniform dispersion. In addition, because elastin is a cross-linked structure, the time course of release of soluble peptides may not be linear with respect to the rate of hydrolytic scission of peptide bonds. Another complicating feature of assays for elastolytic enzymes is the fact that the enzymes or proenzymes, if any, may bind to the insoluble substrate.

In this paper we describe a simple, sensitive radioactive assay for elastolytic activity that may be used both for screening and kinetic studies. We also describe how this assay, used in conjunction with pretreatment of the elastin substrate with sodium dodecyl sulfate, may reveal information concerning the organization of the polypeptide chain in the cross-linked domains of elastin.

The basis of this method is that mature elastin contains cross-links probably distributed with some regularity throughout the fiber, and that some of these cross-links (aldol, desmosines, isodesmosines, *etc.*) are reducible by $NaBH_4$ (see, for instance, review by Gallop *et al.*[8]). If tritiated $NaBH_4$ is used, the elastin becomes labeled extensively, and provides a means of quantifying elastolysis by appearance of radioactivity in the soluble phase.

Another radioactive assay depending mainly on the iodination of elastin was employed by Robert and Robert[5].

MATERIALS AND METHODS

Elastin was prepared from bovine ligamentum nuchae[9] and finely powdered by use of a "Micro Mill" apparatus obtained from the Lab Apparatus Co., Cleveland, Ohio. The ground material was passed through a sieve to give a mesh size of 200. Reduction was carried out as follows[10]. Elastin (100 mg) was suspended in 5 ml of water and the pH of the mixture adjusted to 8.9 by addition of Na_2CO_3. Tritiated $NaBH_4$ (New England Nuclear) diluted with normal $NaBH_4$ (45–50 mg of the mixture was employed with specific activity of $8.5 \cdot 10^6$ dpm/μmole) was added, and the mixture stirred for 1 h at room temperature. The reaction was terminated by addition of acetic acid to pH 3.0. The modified elastin was removed by centrifugation and washed with distilled water until the supernate no longer showed radioactivity. The elastin was then suspended in water and lyophilized. As an example, one mg of the treated elastin, standardized on the basis of its nitrogen content determined by Kjeldahl analysis, yielded $2.6 \cdot 10^5$ cpm; there was some variation among different preparations.

Elastases of several origins have been used in these studies. The enzyme used principally was purified crystalline porcine pancreatic elastase (60 units/mg protein) purchased from Worthington Biochemical Corp. Other elastolytic preparations were used merely to establish the general applicability of the radioactive assay. These

included preparations made from *Clostridium histolyticum* (Takahashi *et al.*[11]), dog pancreatic juice, dog pancreatic tissue, and from the hepatopancreases of the fiddler crab and of the fresh-water crayfish (Takahashi and Seifter[12]). Preparation and characteristics of these enzymes will be described elsewhere. Pronase was purchased from the Kaken Co. of Tokyo, trypsin (3 times crystallized) was purchased from the Sigma Chemical Co., and α-chymotrypsin from Worthington Biochemical Corp.

Elastolytic activity was compared by several assay methods. For this purpose reactions were conducted by a modification of a procedure described by Lamy *et al.*[1]. Into a plastic centrifuge tube (0.5 cm × 5 cm) was placed 0.2 ml of the milled and graded elastin in the form of a 0.5% suspension of 0.05 M Tris buffer of pH 8.0. To this was added 0.2 ml of enzyme solution, and 0.2 ml of 0.05 M Tris buffer of pH 8.0. The mixture was incubated at 37 °C with shaking, and enzyme activity terminated at a desired time by addition of diisopropylphosphorofluoridate to a final concentration of 0.04 M. The mixture was centrifuged at 27 000 for 20 min, and the supernate removed and measured for radioactivity. Radioactivity was measured using a Mark I Liquid Scintillation Counter purchased from Nuclear Chicago; the liquid scintillation mixture used was either Packard-Bray or Biosolve Scintillation mixture. Amino acid analysis was performed using a Technicon amino acid analyzer. Ninhydrin reactivity was determined by the method of Rosen[13], and biuret–Folin reactivity by the method of Lowry *et al.*[14].

RESULTS AND DISCUSSION

In the first series of experiments, 1 mg of tritiated reduced elastin was incubated with 0.005 mg of porcine pancreatic elastase and at various times a sample removed for analysis. Fig. 1 shows the results of one such experiment. (The inherent deviations from linearity, described in the introduction, are noted even in the earlier time periods.) The time at which the elastin is completely solubilized is determined as the earliest time at which maximum radioactivity appears in solution. One may then note in the figure that the rate of solution of the substrate measured by any of the 3 parameters used was approximately linear to the point at which almost 80% of the elastin was dissolved. Beyond the point of complete solubilization of the substrate,

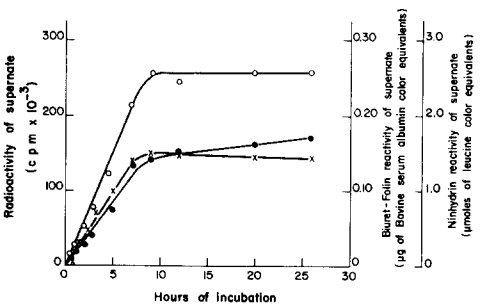

Fig. 1. Time course of elastolysis of reduced tritiated elastin by porcine pancreatic elastase used as described in the text. Extent of reaction determined in supernate by: ○, radioactivity; ●, ninhydrin reactivity; and ×, reactivity with biuret–Folin reagent. Ratio of enzyme to substrate was 1:200 (w/w).

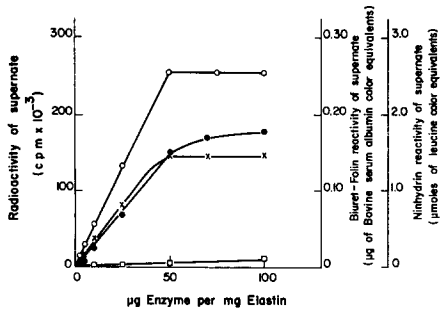

Fig. 2. Digestion of reduced tritiated elastin by various amounts of enzyme in one hour's time under conditions described in the text. Extent of reaction determined in the supernate: ○, radioactivity measured when enzyme used was pancreatic elastase; ●, ninhydrin reactivity when the enzyme used was pancreatic elastase; and ×, reactivity with biuret–Folin reagent when enzyme used was pancreatic elastase; □, Radioactivity measured when the enzyme was either trypsin or chymotrypsin.

the increase of ninhydrin reactivity merely indicates that the enzyme continued to act on the peptide fragments in solution. Decrease in biuret–Folin reactivity in this late period may occur to a minor extent because the soluble peptides may continue to be cleaved. The different slopes for the 3 parameters in the "linear" portions of the curves reflect the circumstance that the measured parameters are not always directly related in time. Thus in all comparative assays reported here one must consider the following differences in measurement. Radioactivity released to the soluble phase undergoes no further change; however, peptides released may change either in ninhydrin or biuret–Folin reactivity depending on whether the elastase is also released from its binding to the insoluble phase. This is to say that elastase, if released, may continue to degrade solubilized peptides with the consequences already noted. As seen in Fig. 1, this circumstance alone makes the radioactive assay the most reliable measure of proteolysis among the 3 assays used.

Fig. 2 shows the effect of varying enzyme concentration on elastolysis of tritiated reduced elastin. One may note that 1 mg of elastin was solubilized in 1 h by 0.05 mg of porcine pancreatic elastase, and again the most sensitive and perhaps most reliable assay was the measurement of released radioactivity. The figure

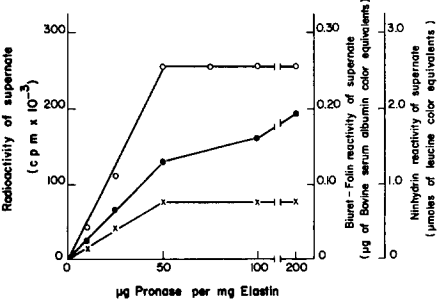

Fig. 3. Digestion of reduced tritiated elastin by various amounts of pronase in one hour's time under conditions described in the text. Extent of reaction determined in the supernate: ○, radioactivity; ●, ninhydrin reactivity; and ×, reactivity with biuret–Folin reagent.

includes a plot of the release of radioactivity by trypsin or α-chymotrypsin, showing that the amount of measured elastolysis by either of these enzymes was extremely low, in keeping with the known incapacity of these enzymes to digest elastin. The solubilization of elastin in absence of any enzyme was virtually zero.

Pronase preparations are known to have elastolytic activity. Fig. 3 shows the results of an experiment in which varying amounts of pronase were allowed to act for 1 h on 1 mg of reduced tritiated elastin. Solubilization of the substrate was complete in this time when the enzyme: substrate ratio was 1:20, but again the ninhydrin reactivity continued to increase perhaps for the reasons already noted.

Experiments with elastolytic enzymes obtained from dog pancreatic juice or dog pancreas, from the hepatopancreases of the fiddler crab or fresh water crayfish, and from *C. histolyticum* all gave similar curves to those shown for the porcine pancreatic elastase.

Experiments were then performed to determine the effects on the radioactive assay of pretreatment of the reduced tritiated substrate with sodium dodecyl sulfate. Relatively low concentrations of this agent have been shown to enhance elastolysis by pancreatic elastase[15,16], whereas concentrations in the range of 0.03% inhibit the enzymatic action against a synthetic substrate[17]. Evidence has been presented to show that the reagent becomes adsorbed to insoluble elastin; and in the case of the oxalic acid solubilized α-elastin, probably causes conformational changes[16]. Among the domains of elastin that might be modified are those "polyalanine"-like regions surrounding the cross-links. These regions in fact may bear the small amount of α-helical structure found in elastin, and accordingly could be disrupted by the detergent. Other highly prevalent domains of elastin are hydrophobic and are likely to be affected by the reagent; in fact, formation of new regions of α-helix could be promoted.

In the present experiments, reduced tritiated elastin was treated with 0.5% sodium dodecyl sulfate according to the method of Kagan *et al.*[16], and the suspension then centrifuged. The elastin (1 mg) was suspended in Tris buffer and assayed as described in a previous section.

Fig. 4 compares the time course of activity of pancreatic elastase on reduced tritiated elastin with the activity on reduced tritiated elastin that had been treated previously with sodium dodecyl sulfate; it shows that the early rates in the radioactive assay in the latter case were approximately twice those in the former (Fig. 4a). In the same experiments the rates of appearance of ninhydrin-reactive (Fig. 4b) and biuret–Folin reactive (Fig. 4c) materials in the supernates also were greater in the case of sodium dodecyl sulfate-treated substrate; however, there were significant differences among the parameters measured. Table I summarizes the "early" rates of elastolysis determined from the almost linear portions of the curves in Fig. 4. The data demonstrate that pretreatment of elastin with sodium dodecyl sulfate caused a subsequent greater increase in the number of proteolytic scissions (ninhydrin reactivity) as compared with degree of solubilization. Table II demonstrates that at the point of 50% solubilization of the elastin as determined by radioactivity in the supernate, pretreatment with sodium dodecyl sulfate caused an increase of 1.4 times in the number of proteolytic scissions that had occurred in the absence of such pretreatment. However, at this point a decrease in the biuret–Folin reactivity of the supernate occurred. This would appear to indicate that sodium dodecyl sulfate

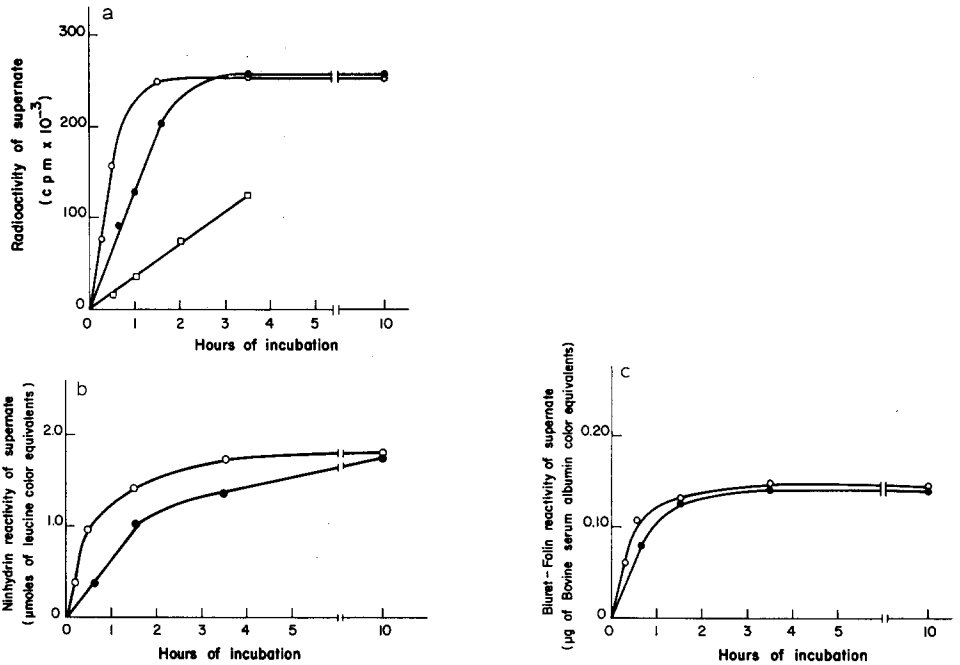

Fig. 4. Time course of elastolysis of reduced tritiated elastin by porcine pancreatic elastase used as described in the text. Curves 4a, 4b and 4c compare the extent of reaction measured by several parameters when the elastin received prior treatment with sodium dodecyl sulfate as described with extent when the elastin had no such prior treatment: ○, prior treatment with sodium dodecyl sulfate; ●, no prior treatment with sodium dodecyl sulfate; and, in curve 4a, □, when the elastolysis was conducted on tritiated elastin that had been pretreated with polylysine (Miles-Yeda Inc.), in a ratio of 1 mg elastin with 50 μg of polymer. Ratio of enzyme to substrate in all cases was 1:100 (w/w).

TABLE I

"EARLY" RATES OF ELASTOLYSIS BY PORCINE PANCREATIC ELASTASE OF REDUCED, TRITIATED ELASTIN THAT HAD OR HAD NOT RECEIVED PRETREATMENT WITH SODIUM DODECYL SULFATE

Data obtained from Fig. 4.

Rate measured by	*No sodium dodecyl sulfate*	*Pretreated with sodium dodecyl sulfate*	*Times increase with sodium dodecyl sulfate*
Radioactivity (cpm/min × 10^{-3})	2.13	4.92	2.3
Ninhydrin reactivity (Leu equiv./min)	0.010	0.033	3.3
Biuret–Folin reactivity (albumin color equiv./min)	0.002	0.0037	1.8
			Ratio: plus sodium dodecyl sulfate/minus sodium dodecyl sulfate
(Time in which elastin completely solubilized measured by radioactivity: minutes)	210	108	0.51
(Time in which elastin 50% solubilized, measured by radioactivity: minutes)	60	26	0.43

TABLE II

COMPARISON OF SEVERAL PARAMETERS OF ELASTOLYSIS BY PORCINE PANCREATIC ELASTASE OF REDUCED, TRITIATED ELASTIN THAT HAD OR HAD NOT RECEIVED PRETREATMENT WITH SODIUM DODECYL SULFATE

Comparisons were made at the time when the elastin was completely dissolved as determined by radioactivity and again when the elastin was 50% solubilized.

Parameter measured	*No sodium dodecyl sulfate*		*Pretreated with sodium dodecyl sulfate*		*Ratio: plus sodium dodecyl sulfate/minus sodium dodecyl sulfate*	
	50%	*100%*	*50%*	*100%*	*50%*	*100%*
Radioactivity (cpm × 10^{-3})	128	256	128	256	1.0	1.0
Ninhydrin reactivity (Leu equiv.)	0.63	1.37	0.89	1.48	1.4	1.1
Biuret–Folin reactivity (albumin color equiv.)	1.35	1.40	0.88	1.35	0.7	1.0

promoted more proteolytic scissions with no corresponding increase in degree of solubilization (to the 50% point), and that the peptides appearing in the supernate accordingly must have been smaller or, alternatively, were smaller but were interjoined by retained cross-links. One may infer, then, that sodium dodecyl sulfate exposed portions of the elastin structure causing an increase in the rate of proteolysis. Preliminary study of the peptides produced suggest that the cross-linked regions may have structures particularly sensitive to the effects of sodium dodecyl sulfate, and this perhaps is consistent with the occurrence of regions dominated by –Ala–Ala– and –Ala–Ala–Ala–Ala– sequences[18]. These data add to the considerations of Kagan *et al.*[16] about the enhancing effects on elastase of sodium dodecyl sulfate.

An experiment also was performed with a positively charged polyelectrolyte, namely polylysine, shown by Gertler[19] to inhibit elastolysis. Fig. 4a contains a curve showing the inhibitory effect of polylysine demonstrated by use of the new assay procedure described here.

ACKNOWLEDGEMENTS

The authors are indebted to Drs Paul M. Gallop and Carl Franzblau for helpful suggestions and criticisms. This research was supported by grants AM 11013 and AM 03172 from the U.S. Public Health Service.

REFERENCES

1 Lamy, F., Craig, C. P. and Tauber, S. (1961) *J. Biol. Chem.* 236, 86–91
2 Quinn, R. S. and Blout, E. R. (1970) *Biochem. Biophys. Res. Commun.* 40, 328–333
3 Naughton, M. A. and Sanger, F. (1961) *Biochem. J.* 78, 156–163
4 Sachar, L. A., Winter, K. K., Sicher, N. and Frankel, S. (1955) *Proc. Soc. Exp. Biol.* 90, 323–326
5 Robert, B. and Robert, L. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), pp. 665–670, Academic Press, New York, N.Y.
6 Thompson, R. C. and Blout, E. R. (1973) *Biochemistry* 12, 51–56
7 Thompson, R. C. and Blout, E. R. (1973) *Biochemistry* 12, 57–65
8 Gallop, P. M., Blumenfeld, O. O. and Seifter, S. (1972) *Annu. Rev. Biochem.* 41, 617–672
9 Partridge, S. M., Davis, H. F. and Adair, G. S. (1955) *Biochem. J.* 61, 11–21
10 Paz, M. A., Gallop, P. M., Blumenfeld, O. O., Henson, E. and Seifter, S. (1971) *Biochem. Biophys. Res. Commun.* 43, 289–297

11 Takahashi, S., Seifter, S. and Binder, M. (1970) *Biochem. Biophys. Res. Commun.* 39, 1058–1064
12 Takahashi, S. and Seifter, S. (1972) *Fed. Proc.* 31, 912 Abs.
13 Rosen, H. (1957) *Arch. Biochem. Biophys.* 57, 10–15
14 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
15 Hall, D. A. and Czerkawski, J. W. (1961) *Biochem. J.* 80, 128–134
16 Kagan, H. M., Crombie, G., Jordan, R. and Franzblau, C. (1972) *Biochemistry* 11, 3412–3418
17 Visser, L. and Blout, E. R. (1971) *Biochemistry* 10, 743–752
18 Sandberg, L. B., Weissman, N. and Gray, W. R. (1971) *Biochemistry* 10, 52–56
19 Gertler, A. (1971) *Eur. J. Biochem.* 20, 541–546