Biochimica et Biophysica Acta, 429 (1976) 950—953

BBA 67780

CHARACTERIZATION OF AGAROSE-BOUND TRYPSIN

BERT WALTER

Department of Biochemistry, Brandeis University, Waltham, Mass. 02154 (U.S.A.)

(Received October 16th, 1975)

Summary

Agarose-bound trypsin (EC 3.4.21.4) was prepared and its properties were compared with those of soluble trypsin. The bound form of the enzyme was found to be equally available to large and small molecular weight substrates as the soluble form. In addition, the bound form of the enzyme showed the same specificity towards protein substrates as the soluble enzyme. However, the agarose-bound trypsin showed greater stability than the soluble trypsin to denaturing conditions for prolonged period of time.

Introduction

There have been conflicting reports on the comparative properties of free and matrix-bound enzymes [1—3] and this work was undertaken with the purpose of establishing some comparative data on one well characterized enzyme, namely trypsin (EC 3.4.21.4), in the soluble form and bound to agarose. The specific properties investigated were (1) relative activity towards large and small substrates; (2) specificity of cleavage in protein substrates, and (3) relative activity under denaturing conditions.

Materials and Methods

Bovine pancreatic trypsin (Worthington) was covalently bound to agarose A-15 M, 50—100 mesh (Bio-Rad) by the CNBr procedure of Axén et al. [4]; the binding was conducted in the presence of 10 mM $CaCl_2$ to reduce trypsin autolysis. The amount of trypsin covalently attached to agarose was determined by amino acid analysis [5,6] on Beckman Model 120C amino acid analyzer after hydrolysis of 1 ml of settled agarose-trypsin in 6 M HCl for 24 h at 110°C.

Soluble and matrix-bound trypsin activity was assayed titrimetrically with a Radiometer pH-stat system using 0.01 M KOH as a titrant; the temperature was

maintained at 25°C with a Haake temperature control bath. A typical assay consisted of 10 ml of 15 mM *p*-tosylarginine methyl ester (TosArgOMe) (Sigma), pH 8.0, and 0.006—0.009 μmol of soluble trypsin or agarose-trypsin having a comparable amount of active trypsin. The total amount of active trypsin per given quantity of agarose was determined from a standard curve constructed using the rate of TosArgOMe hydrolysis by soluble trypsin as a function of enzyme concentration. In addition, proteolytic action of trypsin and agarose-trypsin on proteins was also monitored titrimetrically. In all cases, the slope of the tangent to the initial part of the titration curve was taken as the rate of substrate hydrolysis.

Results and Discussion

Specific activity and relative activity towards p-tosyl arginine methyl ester, casein and insoluble protein substrates

A total of 0.01 μmol of enzymatically active trypsin was bound per 1 ml of settled agarose based on the rate of TosArgOMe hydrolysis; this activity accounted for 20% of the total amount of bound enzyme. In the subsequent discussion, reference to a given quantity of agarose-trypsin refers only to the amount of active trypsin as determined by the rate of TosArgOMe hydrolysis.

When agarose-bound trypsin and soluble trypsin of comparable activity levels were allowed to act on TosArgOMe and bovine casein in parallel experiments, the ratio of the rate of TosArgOMe hydrolysis to casein hydrolysis (TosArgOMe/casein) was found to be 13.9 for both the matrix-bound and soluble form of the enzyme. This clearly shows that the agarose-bound enzyme is equally available to the large protein substrate (casein approx. 25000 mol.wt.) as it is to the small substrate (TosArgOMe approx. 379 mol.wt.). On the other hand, when soluble and agarose-bound trypsin were allowed to act on insoluble denatured proteins, a marked difference in activity was observed. Whereas no insoluble substrate was found to be resistant to soluble trypsin, the agarose-bound enzyme showed little or no activity towards insoluble substrates.

Specificity of free and agarose-bound trypsin

Using several protein substrates (bovine casein, performic acid oxidized RNAase A and horse hemoglobin), the following survey was conducted. After exhaustive exposure of substrate to soluble trypsin (where proton production subsided as determined by pH-stat assay), the solution was heated to denature the trypsin, and agarose-bound trypsin was introduced at 25°C. The opposite experiment was carried out too; after exposure to agarose-bound trypsin, the bound enzyme was removed, the solution heated and soluble trypsin was introduced at 25°C. A typical set of results, where casein was used as a substrate, is illustrated in Fig. 1. In no case was significant further hydrolysis of substrate observed after the second introduction of enzyme. This suggests that the same bonds must be cleaved by both enzyme preparations. In the case of oxidized ribonuclease A, the peptides produced by the action of agarose-trypsin were compared to those produced by soluble trypsin; the resulting peptide profiles upon fractionation on phosphocellulose were found to be identical for both forms of the enzyme. Amino acid analysis showed that agarose-trypsin cleaved

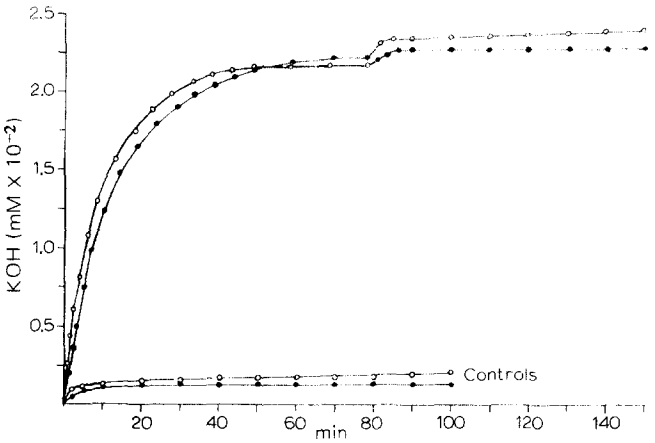

Fig. 1. Exhaustive digestion of bovine casein by soluble trypsin (○) and agarose-trypsin (●). 10 ml of 0.2% casein solution was digested at 25°C with 0.01 μmol of soluble or agarose-bound trypsin followed by heating the solution to 70°C for 15 min, cooling to 25°C and introducing 0.01 μmol of agarose-trypsin or soluble trypsin, respectively. Control experiments contained 0.01 μmol of either soluble trypsin or agarose-trypsin in the absence of the substrate casein. Digestion was monitored at pH 8.0 by a Radiometer pH-stat.

only at lysine and arginine residues as reported by Chin and Wold [7] where eight lysine- and four arginine-containing peptides were found in each case with the expected amino acid content [8]. A comparison of the rate and extent of activation of chymotrypsinogen and trypsinogen by the two forms of the enzyme also gave identical results.

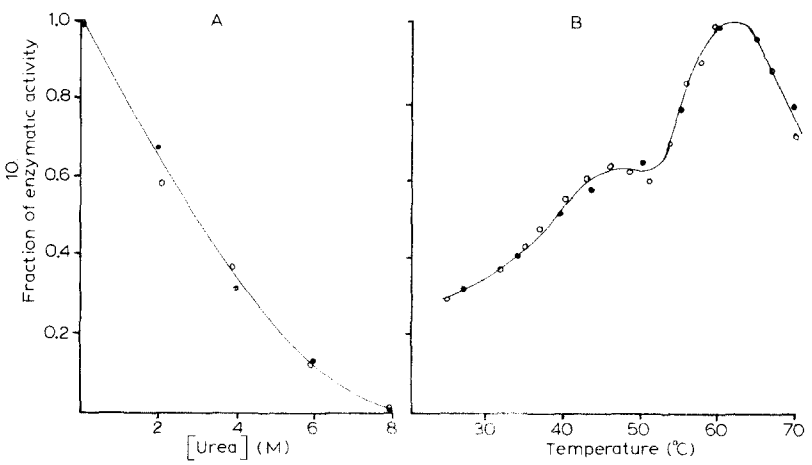

Fig. 2. (A) Activity profile of soluble trypsin (○) and agarose-trypsin (●) as a function of urea concentration. Enzymatic activity was determined by introducing 0.006—0.009 μmol of soluble or agarose-bound trypsin into 10 ml of 15 mM TosArgOMe solution, with the appropriate urea concentration, and monitoring the initial hydrolysis rate at 25°C, pH 8.0, with a Radiometer pH-stat system. (B) Activity profile of soluble trypsin (○) and agarose-trypsin (●) as a function of temperature. A 0.006—0.009 μmol of soluble or agarose-bound trypsin was preincubated for 3 min in 5.0 ml of water at a given temperature and 5.0 ml of substrate solution, maintained at the same temperature was introduced into the preincubated trypsin solution to give a 10 mM TosArgOMe concentration. The rate of substrate hydrolysis was determined as described in A. All activities are expressed relative to maximal activity at 62°C.

Effect of temperature and urea on free and agarose-bound trypsin

The urea denaturation profiles for agarose-trypsin and soluble trypsin are illustrated in Fig. 2A. Both species show identical profiles where enzymatic activity decreases nearly linearly with increasing urea concentration and total inactivation is observed in 8 M urea. The temperature profiles for both forms of the enzyme in the presence of 10 mM $CaCl_2$ were also examined and the results are summarized in Fig. 2B. Both species show similar temperature profiles where specific activity increases with increasing temperature and optimum activity is observed at 62°C. At temperatures above 62°C inactivation due to heat denaturation predominates and loss of activity is observed; these results are in good agreement with the observations made by Sipos and Merkel [9] for trypsin activity in the presence of calcium as a function of temperature.

Stability of agarose-bound trypsin

All the observations on the stability of agarose-trypsin upon storage under different conditions or under exposure to severe conditions for extended periods of time are constant with an increased stability over soluble trypsin. Agarose-trypsin maintained constant specific activity after repeated use over a 9-month period when stored at 5°C at neutral pH. In 4 M urea, agarose-trypsin maintained constant activity for 4 h at 25°C where activity was 50% of that observed in the absence of urea; upon removal of urea, full activity was recovered. In addition, the bound enzyme maintained a constant specific activity at 50°C for a duration of 5 h, making it feasible to digest trypsin-resistant proteins at elevated temperatures. The high stability of agarose-trypsin is in agreement with earlier observations [1,3] and is most likely due to protection against autolysis which is prevented when individual trypsin molecules are kept apart on the agarose matrix.

It is concluded that active trypsin attached to agarose beads behaves like soluble trypsin in terms of specificity, availability to soluble substrates and behavior to denaturing conditions for short periods of time when autolysis is minimized. The agarose-bound trypsin is distinctly different from soluble trypsin in its inability to interact with insoluble substrates and this property is undoubtedly reflected in the increased stability of the agarose-bound enzyme.

Acknowledgement

This work was supported in part by a U.S.P.H. research grant (GM 15053) to Dr. F. Wold, Department of Biochemistry, at the University of Minnesota in whose laboratory these experiments were conducted.

References

1 Knight, R.J. and Light, A. (1974) Arch. Biochem. Biophys. 160, 377—386
2 Axén, R. and Ernbach, S. (1971) Eur. J. Biochem. 18, 351—360
3 Gable, D., Vretbald, P., Axén, R. and Porath, J. (1970) Biochim. Biophys. Acta 214, 561—563
4 Axén, R., Porath, J. and Ernbach, S. (1967) Nature 214, 1302—1304
5 Moore, S., Spackman, D.H. and Stein, W.H. (1958) Anal. Chem. 30, 1185—1206
6 Benson, Jr., J.V. and Patterson, J.A. (1965) Anal. Chem. 37, 1108—1110
7 Chin, C.Q. and Wold, F. (1972) Anal. Biochem. 46, 585—593
8 Hirs, C.H.W., Moore, S. and Stein, W.H. (1956) J. Biol. Chem. 219, 623—642
9 Sipos, T. and Merkel, J.A. (1970) Biochemistry 9, 2766—2775