PARTITION OF TRYPSIN IN TWO-PHASE SYSTEMS CONTAINING A DIAMIDINO-α, ω-DIPHENYLCARBAMYL POLY (ETHYLENE GLYCOL) AS COMPETITIVE INHIBITOR OF TRYPSIN

Georges TAKERKART*, Emile SEGARD** and Michel MONSIGNY*‡

*Centre de Biophysique Moléculaire, CNRS, et Laboratoire de Chimie Biologique, UER Sciences 45045 Orleans-Cedex, France, and
**Centre d'Etudes Cryogéniques de L'Air Liquide 38360 Sassenage, France

Received 18 February 1974

1. Introduction

The purification of biological macromolecules by partition in aqueous two-phase systems is based mainly on physico-chemical properties of systems and of macromolecules. This is well illustrated by the system obtained by mixing aqueous solutions of dextran and poly (ethylene glycol) (PEG). Partition of macromolecules in this system depends namely on polyelectrolytes and salts concentration [1,2]. Furthermore, when charged poly(ethylene glycol) is added, partition depends also on the pH of the system, and proteins can be extracted according to their isoelectric points [3,4]. Interactions involved in the process of partition are not specific and a mixture of proteins is often obtained in each phase of the system. Therefore, it would be of interest to introduce highly specific ligands confined to one of the phases in order to purify macromolecules selectively by affinity.

For enzyme purification, the interacting compound may be a highly specific competitive inhibitor attached to PEG, since it is known that, in dextran/PEG systems, PEG is essentially in the upper phase. As a model, p-aminobenzamidine (PAB), a strong inhibitor of trypsin, was attached to PEG and new macro-inhibitors of trypsin were obtained [5]. We describe here the effect of one of them, PEG 9000 PAB, on the partition of trypsin in dextran/PEG 9000 two-phase system.

2. Materials and methods

Dextran T 500 (weight average mol.wt. ($\bar{M}_w$) = 450 000 and number average mol.wt. ($\bar{M}_n$) = 194 000) was supplied by Pharmacia, Uppsala, Sweden. Poly(ethylene glycol) (PEG 9000; $\bar{M}_w$ = 9000) was obtained from Fluka. Diamidino-α,ω-diphenylcarbamyl-poly (ethylene glycol) (PEGPAB); $\bar{M}_w \cong$ 9000) was prepared as described earlier [5] (diamidino-α,ω,-diphenylcarbamyl-poly (ethylene glycol). Trypsin, chymotrypsin, DL-benzoylarginine-*p*-nitroanilide (BAPNA) and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were obtained from Sigma.

2.1. *Phase diagrams*

The binodial for the dextran–PEG–water and for the dextran–PEG/PEGPAB (3:1)–water systems was obtained according to Johansson [3]. Water was added to mixtures of PEG and dextran solutions at room temperature until phase transition occurred.

2.2. *Two-phase systems*

These were prepared at room temperature in 0.05 M Tris–HCl buffer pH 8.0. System (A) was obtained by mixing 20% (w/v) solutions of dextran and PEG

‡ To whom inquiries should be addressed.

Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester.

North-Holland Publishing Company – Amsterdam

with buffered enzyme solutions. System (B) was obtained as system (A) but with PEG/PEGPAB (3:1). This ratio was selected to obtain full complexation of trypsin in regard to K_i value of PEG 9000 PAB ($2.0\ 10^{-5}$ M [5]) and concentration of trypsin in the system (10^{-4} M). Increasing concentration of PEGPAB is not useful since solubility of trypsin in the upper phase is limited. The systems were shaken and then centrifuged for 5 min at 1500 *g*. The phase volume ratio is about 2.

2.3. *Determination of partition coefficients*

The partition coefficient of PEG 9000 was determined by evaporating to dryness an aliquot of the upper phase of system (A). The residue was further dried in vacuo over P_2O_5 to constant weight. The partition coefficient of PEG 9000 PAB was determined by measuring the absorbance of each phase at 270 nm with a Zeiss spectrophotometer PMQ II.

2.4. *Determination of trypsin and chymotrypsin activity*

Trypsin assays were carried out with DL-BAPNA as substrate [6,7]. Chymotrypsin assays were carried out with ATEE as substrate [8]. All measurements were made at 25°C using a Beckman Acta III spectrophotometer.

2.5. *Partition of trypsin and chymotrypsin*

Partitions of trypsin and chymotrypsin were studied by measuring enzymic activities in the upper phase and then expressing them in % of total introduced enzyme activities. PEGPAB does not affect the measurement of trypsin activity since its concentration in the sample cuvette (0.01 mM) is very low in regard to that of substrate (1 mM).

3. Results and discussion

3.1. *Phase diagram*

Fig. 1 shows the phase diagrams for system (A) and system (B). The binodial obtained when PEG PAB is added instead of PEG is close to that obtained with unsubstituted PEG. This indicates that the molecular weight of PEG has not changed during substitution [1] in good agreement with our previous results [5].

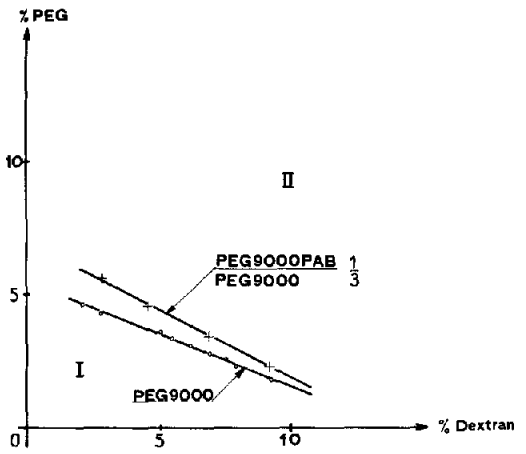

Fig. 1. Phase diagrams for systems PEG9000–dextran–water and PEG9000/PEG9000PAB (3:1)–dextran–water. Part of the binodial separating 1-phase (I) region and the 2-phase region is shown.

3.2. *Partition coefficients of polymers*

The determination of PEG 9000 partition coefficient was conducted as described above because no precipitation was obtained when adding ethanol to an upper phase of system (A). This demonstrates the absence of dextran in the upper phase. The determined value of PEG 9000 partition coefficient is 1.8. In system (B), the ratio of PEGPAB concentrations between the upper and lower phases is 8.6 as determined by spectrophotometric measurements. This value is quite different from that of unsubstituted PEG and indicates an enhancement of the affinity of PEG for the upper phase when *p*-aminobenzamidine is attached to each end of the chain.

3.3. *Trypsin partition*

Concentration of trypsin in systems (A) and (B) was 10^{-4} M. In system (A), recovery of trypsin activity in the upper phase was 40% of the total activity. Partition coefficient of trypsin in that system was calculated to be 0.33. In system (B), recovery of trypsin activity in the upper phase was 92% of the total activity. In that system the trypsin partition coefficient was 5.7. It appears from these values that trypsin concentration in the upper phase is increased as much as 2.3 times when PEGPAB is added to the two-phase system. In other words, there is a 17-fold

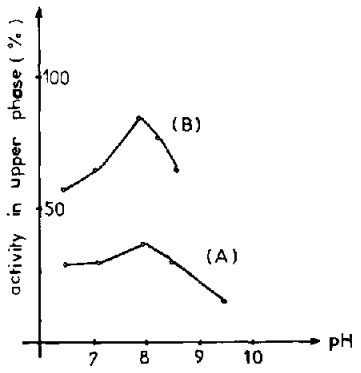

Fig. 2. pH dependance of trypsin partition in dextran–PEG 9000 system (A) and in dextran–PEG9000/PEG9000PAB (3:1) system (B). Trypsin activity is expressed as percentage of total added activity.

increase of the trypsin partition coefficient when a specific trypsin inhibitor is attached to PEG. Thus, the partition coefficient of trypsin is close to that of PEGPAB showing a strong interaction between enzyme and inhibitor.

The pH dependance of trypsin partition in systems (A) and (B) is shown in fig. 2. In both systems, the trypsin partition coefficient exhibits a maximum at pH 8.0. The ionic strength dependence of trypsin partition coefficient is shown in table 1. In system (A), the recovery of trypsin in the upper phase decreased slightly when Tris concentration increased. In system (B), no significant effect could be noted.

Finally, specificity of the extraction of trypsin was demonstrated by investigating partition of chymotrypsin in the same systems. When chymotrypsin was added as a unique protein to systems (A) and (B), recovery of activity in the upper phase was respectively 20% and 28% of total introduced activity. Thus, the chymotrypsin partition coefficients are 0.12 and 0.19 respectively, that is, a 1.5 time increase in system (B). This is to be compared with the 17 times increase obtained with trypsin. Upon addition of a mixture of trypsin and chymotrypsin to systems (A) and (B), the trypsin partition coefficient became 0.26 and 3.35 respectively whereas those of chymotrypsin became 0.11 and 0.15. Though there is a small decrease in trypsin partition coefficients values, PEGPAB still exhibits a highly specific effect.

Table 1
Ionic strength (μ) dependence of trypsin partition in dextran–PEG9000 (system A) and in dextran–PEG9000/PEG9000PAB (3:1) (system B). Tris–HCl buffer pH 8.0

	Trypsin activity in upper phase (in % of total activity)	
μ	System (A)	System (B)
0.05	39	89
0.1	36	77
0.2	35	89

This behaviour is remarkable in consideration of very similar properties of trypsin and chymotrypsin.

This effect seems to be applicable to other interacting systems and its usefulness as a step in macromolecules purification is under investigation.

Acknowledgement

This work was partly supported by a grant from L'Air Liquide Co.

This work has been registered: French patents no. 72.45695 on December 21, 1972 and no. 73.42320 on November 28, 1973.

References

[1] Albertsson, P. A. (1971) Partition of cell particles and macromolecules, 2nd edn, Wiley Interscience, New York.
[2] Walter, H., Garza, R. and Coyle, R. P. (1968) Biochim. Biophys. Acta 156, 411–413.
[3] Johansson, G. (1970) Biochim. Biophys. Acta 222, 381–389.
[4] Johansson, G., Hartman, A. and Albertsson, P. A. (1973) Europ. J. Biochem. 33, 379–386.
[5] Takerkart, G., Segard, E. and Monsigny, M., unpublished results.
[6] Erlanger, B. F., Kokowsky, N. and Cohen, W. (1961) Arch. Biochem. Biophys. 95, 271–278.
[7] Mares – Guia, M. and Shaw, E. (1965) J. Biol. Chem. 240, 1579–1585.
[8] Schwert, G. W. and Takenaka, Y. (1965) Biochim. Biophys. Acta 16, 570–575.