*Biochimica et Biophysica Acta*, 659 (1981) 370—377
Elsevier/North-Holland Biomedical Press

BBA 69274

# FURTHER PURIFICATION AND CHARACTERIZATION OF CASEIN KINASES FROM HUMAN ERYTHROCYTE HEMOLYSATE

## EFFECT OF TRITON X-100

G. CLARI and V. MORET *

*Institute of Biological Chemistry, University of Padova, Via Marzolo 3, 35100 Padova (Italy)*

(Received October 14th, 1980)

*Key words: Casein kinase; Triton X-100; Hemolysis; (Human erythrocyte)*

## Summary

Two cyclic AMP-independent casein kinases can be isolated from human erythrocyte hemolysate, one of which (referred to as 'casein kinase S') phosphorylates only serine residues of whole commercial casein, while the other (referred to as 'casein kinase TS') phosphorylates both serine and threonine residues of the same substrate. Moreover, the casein kinase S, unlike casein kinase TS, is able to phosphorylate the erythrocyte membrane proteins.

The present paper deals with the further characterization of casein kinase S, freed from histone kinase activity by DEAE and subsequent phosphocellulose chromatography of the crude hemolysate in the presence of 0.2% Triton X-100. In particular, cytosol casein kinase S exhibits some physico-chemical and catalytic properties identical to those of the membrane-bound casein kinase, solubilised and purified as previously described. Both casein kinases display the same chromatographic behaviour, the same Sepharose elution volume, the same optimal pH range, the same $K_m$ for casein and ATP, the same response to NaCl, $MgCl_2$ and $CaCl_2$, and the same ability to phosphorylate serine but not threonine residues of $\beta$-casein.

---

The multiple protein kinases of human erythrocytes, located both in the cytosol and in the membrane structures, are designated casein kinases and histone kinases, being assayed on exogenous proteins such as casein(s) and histones.

* To whom correspondence should be addressed.

A previous paper [1] had shown that the cytosol casein kinases of human erythrocytes are resolved by Sepharose 6B gel filtration at high ionic strength into two fractions, differing from each other in catalytic properties: i.e., the less retarted fraction (referred to as 'TS') phosphorylates both threonine and serine residues of whole casein, while the more retarded fraction (referred to as 'S') phosphorylates only serine residues of casein. Moreover, only this latter fraction S, unlike the fraction TS, has been found to be able to phosphorylate the membrane proteins [2].

Since such inhomogeneous fraction S displayed, in addition to casein kinase, cyclic AMP-independent histone kinase activity, the further purification and characterization of these enzymes was obviously required in order to gain a better knowledge of their relationship with multiple phosphorylatable membrane proteins.

Such an objective, however, has been to date hampered by the marked instability exhibited by casein kinase when the Sepharose fraction was submitted to conventional chromatographic procedures.

The present paper shows that the addition of 0.2% Triton X-100 to the initial crude hemolysate and to the buffered solutions used during the purification steps, allows us to obtain a quite stable and more purified preparation of the cytosol casein kinase S, freed from histone kinase and casein kinase TS.

Cytosol casein kinase S exhibits quite similar properties to those of the casein kinase solubilized and purified from the membranes as previously described [3].

## Methods

Human erythrocytes were obtained from the hospital blood bank (Padova). The erythrocytes, collected by centrifugation, were washed three times with isotonic phosphate buffer (pH 8) and then lysed according to the procedure of Dodge et al. [4], except that solutions contained 0.05 mM phenylmethylsulfonyl fluoride (PMSF).

*DEAE-cellulose chromatography*. The red hemolysate, after centrifugation at 20 000 × *g* and subsequent addition of 0.2% Triton X-100 (final concentration), was dialysed overnight against buffer A (10 mM Tris-HCl pH 7.2/10 mM mercaptoethanol/0.2% Triton), and then applied to DEAE-cellulose column (3.5 × 13 cm) previously equilibrated with buffer A. The column was first washed free from hemoglobin with 490 ml buffer A and then eluted (80 ml/h) with a 800 ml continuous linear gradient ranging from 0 to 0.8 M NaCl in buffer A. 3.7 ml fractions were collected and assayed for protein kinase activity under the conditions previously described [3].

*P-cellulose chromatography*. The four DEAE peaks displaying protein kinase activity, separately pooled as indicated in Fig. 1, were dialysed against buffer A to remove NaCl and then separately applied to a phosphocellulose column (P-cellulose) (2.2 × 17 cm), previously equilibrated with buffer A. The column was first washed with 80 ml buffer A and then eluted (60 ml/h) with a linear gradient of 400 ml ranging from 0 to 1 M NaCl in buffer A. 3.7 ml fractions were collected and assayed for protein kinase activity as previously described [3].

*Sepharose gel filtration*. Gel filtration of casein kinase fractions resolved by P-cellulose chromatography was performed on a Sepharose 6B column (1.6 × 127 cm) equilibrated and eluted with buffer A containing 0.5 M NaCl (1.8 ml fractions, 6 ml/h).

*Assay of protein kinase activity*. Casein kinase (or histone kinase in the presence and absence of 1 μM cyclic AMP) activity was assayed under conditions previously described [3].

*Other methods*. The protein content in the presence of Triton X-100 was determined following the procedure of Lowry et al. [11] modified by Wang and Smith [5], after vacuum drying of the sample for the removal of interference by mercaptoethanol [6], with bovine serum albumin as standard. Whole casein was prepared from commercial powder (Merck) as previously described [1]. β-Casein was a generous gift from Dr. B. Ribadeau-Dumas.

## Results and Discussion

When the crude red hemolysate, containing 0.2% Triton X-100, is submitted to chromatography on a DEAE column equilibrated with buffer A, the hemoglobin does not adhere to the resin and is eluted from the column with the washing buffer A, while the adsorbed protein kinases are eluted by the subsequent linear gradient of NaCl in buffer A/0.2% Triton X-100.

As shown by the elution profile (Fig. 1), under these conditions four

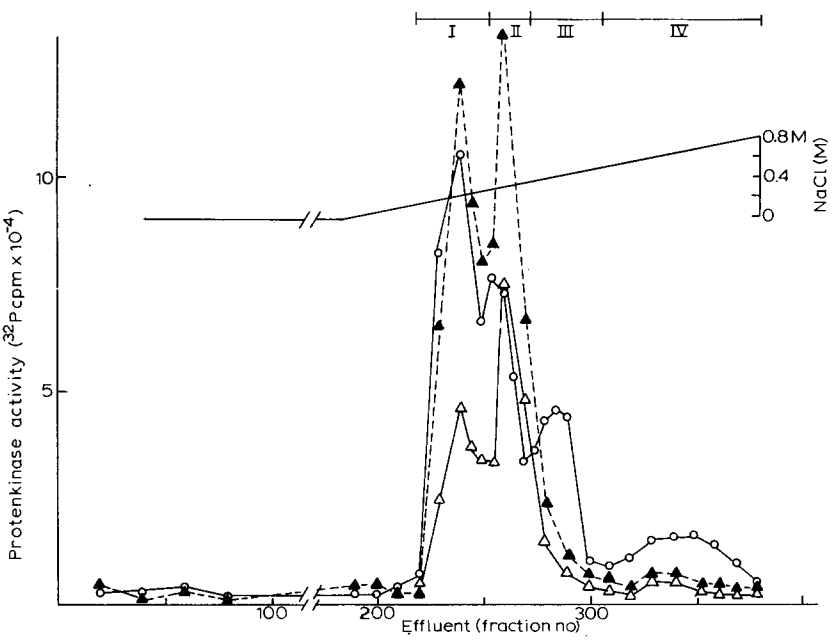


**Fig. 1. DEAE-cellulose chromatography profile of protein kinase activity displayed by the crude hemolysate prepared from 33 ml packed erythrocytes. The crude hemolysate (1000 ml), after addition of 0.2% Triton (final concentration) and after dialysis against buffer A, was submitted to DEAE chromatography. The eluted fractions (100 μl) were assayed for histone kinase activity in the presence of 1 μM cyclic AMP (▲ - - - - - - ▲) and in its absence (△———△) and for casein kinase activity (○———○).**

inhomogeneous protein kinase peaks are eluted by increasing ionic strength: i.e., two major peaks (I and II) eluted by lower ionic strength and displaying both casein kinase and cyclic AMP-dependent histone kinase activity, and two minor peaks (III and IV) eluted by higher ionic strength and displaying preferentially casein kinase activity.

Although the contribution of the different casein kinases to the total activity of the crude hemolysate is difficult to determine, from the data in Table I it is reasonable to assume that under the above conditions the casein kinase activity of the crude initial hemolysate is almost completely recovered into the four quite stable DEAE peaks (Table I), while a marked loss of this activity occurs when the eluting buffer does not contain Triton.

When the four DEAE peaks, separately pooled as indicated in the Fig. 1 and dialysed against the buffer A to remove NaCl, are separately submitted to chromatography on a P-cellulose column equilibrated with buffer A and eluted by linearly increasing concentrations of NaCl in buffer A/0.2% Triton X-100, the elution profiles shown in the Fig. 2a, b, c, and d, respectively, are obtained.

The Fig. 2a shows that histone kinase activity of the major DEAE peak I is resolved by P-cellulose chromatography into two peaks, one of which, containing cyclic AMP-dependent holoenzyme, emerges from the P-cellulose column with washing buffer, while the other, containing cyclic AMP-independent activity (catalytic subunit), is eluted with 0.32 M NaCl. Almost all casein kinase activity is eluted from the P-cellulose column as a single symmetrical peak by 0.67 M NaCl. Only a minor casein kinase fraction is eluted with 0.28 M NaCl, accounting for about 5% of the major peak.

A similar elution profile from P-cellulose column (Fig. 2b) is obtained with the casein kinase activity of the DEAE peak II: i.e., it is completely eluted, as a single symmetrical peak, with 0.67 M NaCl.

The casein kinase activity of the two minor DEAE peaks III and IV is resolved into two small peaks (Fig. 2c and 2d, respectively), the major of which emerges from the P-cellulose column with equilibrating buffer, while the other is eluted from the column by the same ionic strength (0.67 M NaCl) as the casein kinase peak obtained with the major DEAE peaks I and II, and may be

TABLE I

TOTAL CASEIN KINASE ACTIVITY RECOVERED INTO THE FOUR PEAKS OBTAINED BY DEAE CHROMATOGRAPHY OF CRUDE HEMOLYSATE

Casein kinase activity is expressed as cpm $\times 10^{-6}$ transferred from [$\gamma$-$^{32}$P]ATP to casein and referred to the crude hemolysate prepared from 33 ml packed erythrocytes and to the total volume of the four DEAE pooled fractions (Fig. 1). Both the crude hemolysate and the four DEAE fractions, prior to assay, were extensively dialysed against buffer A in order to remove the different salts (phosphate, NaCl). Under these conditions, the rate of casein phosphorylation by all different fractions was linear with respect to the increasing amount of the enzyme used in the assay.

| | |
|---|---|
| Crude hemolysate | 108.0 |
| DEAE peak I | 52.0 |
| DEAE peak II | 19.7 |
| DEAE peak III | 25.6 |
| DEAE peak IV | 9.0 |

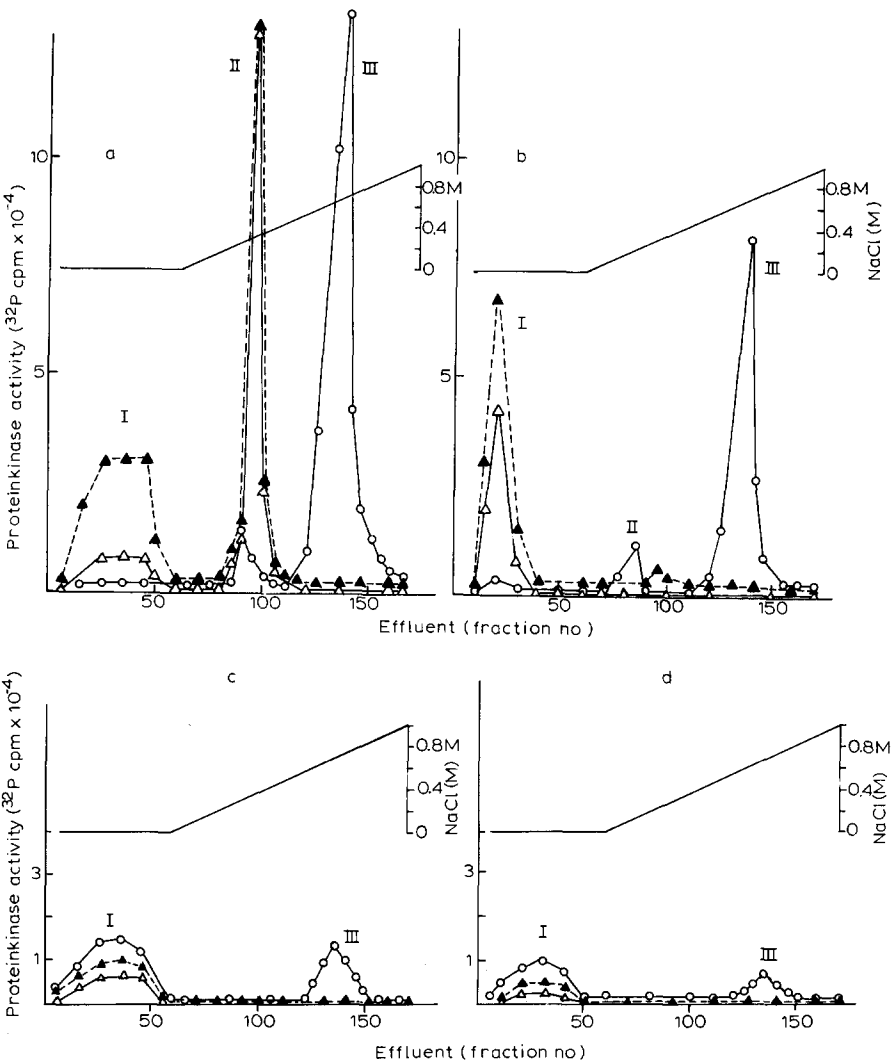


Fig. 2. Phosphocellulose chromatography profile of protein kinase activity displayed by the previously resolved DEAE peaks. (a) DEAE peak I; (b) DEAE peak II; (c) DEAE peak III; (d) DEAE peak IV. The four DEAE peaks, separately pooled as indicated in the Fig. 1, were dialysed against buffer A and then submitted to P-cellulose chromatography. The eluted fractions (100 $\mu$l) were tested for histone kinase activity in the presence of 1 $\mu$M cyclic AMP (▲ - - - - - - ▲) and in its absence (△———△) and for casein kinase activity (○———○).

due, at least in the case of the DEAE peak III, to its contamination by partially overlapping DEAE peak II.

As shown in Table II, the casein kinase activity recovered into the various P-cellulose peaks accounts almost completely for the casein kinase activity of the corresponding DEAE peaks submitted to the P-cellulose chromatography.

All four casein kinase fractions eluted from P-cellulose column with 0.67 M NaCl exhibit:

(1) The same optimal activity between pH 6.8—8.6.

TABLE II

TOTAL CASEIN ACTIVITY RECOVERED INTO THE PEAKS FROM P-CELLULOSE CHROMATOGRAPHY OF THE FOUR DEAE FRACTIONS

Casein kinase activity is expressed as cpm × $10^{-6}$ transferred from [$\gamma$-$^{32}$P]ATP to the casein and referred to the total volume of the different peaks obtained by P-cellulose chromatography from the four DEAE peaks. All fractions, before being assayed, were dialysed against buffer A.

| | DEAE | | | |
|---|---|---|---|---|
| | peak I | peak II | peak III | peak IV |
| Casein kinase eluted with equilibrating buffer (peak I) | — | — | 15.8 | 5.4 |
| Casein kinase eluted with 0.28 M NaCl (peak II) | 2.7 | 2.8 | — | — |
| Casein kinase eluted with 0.67 M NaCl (peak III) | 44.5 | 16.8 | 8.2 | 3.8 |

(2) The same elution profile on Sepharose 6B filtration.

(3) The same apparent $K_m$ for casein (0.16 mg/ml) and for ATP (28 $\mu$M).

(4) The same response to increasing concentrations of NaCl (Fig. 3a), $MgCl_2$ (Fig. 3b) and $CaCl_2$ (Fig. 3c).

Moreover, all four casein kinases phosphorylate only serine residues of whole casein and $\beta$-casein (Fig. 4a).

This identical physico-chemical and catalytic behaviour indicates that the four P-cellulose peaks contain the same casein kinase (referred to as 'casein kinase S'). The enzyme is eluted from the previous DEAE column into multiple peaks, quite likely as a result of aggregation with different cytosolic components which alter its chromatographic properties. The above physico-chemical and catalytic properties of casein kinase S are identical to those of membrane-bound casein kinase, solubilized and purified as previously described [3]. In particular, both casein kinases phosphorylate serine residues of whole casein and $\beta$-casein, but not threonine residues of the same substrates. However, it cannot be ruled out that serine residues phosphorylated by the two casein kinases may be located in different regions of the substrate peptide chain.

The minor casein kinase peak which emerged from the P-cellulose column

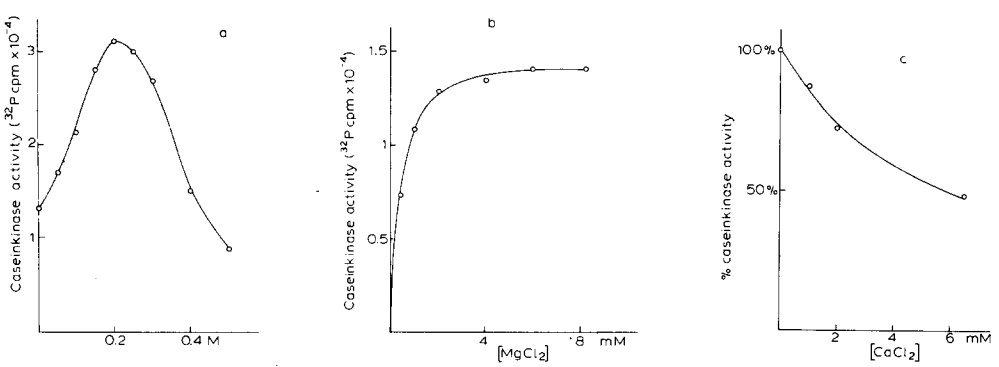


**Fig. 3. Effect of increasing concentrations of NaCl (a), $MgCl_2$ (b) and $CaCl_2$ (c) on the casein kinase activity displayed by each of the four fractions eluted from P-cellulose column with 0.67 M NaCl. The effect of increasing concentrations of $CaCl_2$ was tested in the presence of 4 mM $MgCl_2$.**

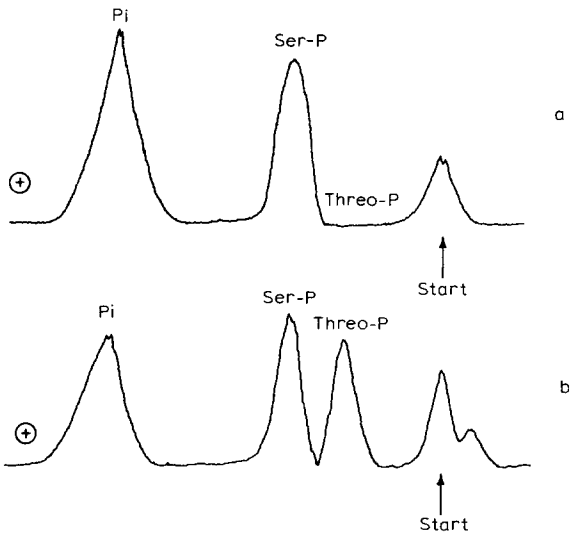


Fig. 4. Electrophoretic pattern of acid hydrolysate of whole casein $^{32}$P-labeled in the presence of [$\gamma$-$^{32}$P]ATP by: (a) each of the four casein kinase peaks (Fig. 2) eluted from the P-cellulose column with 0.67 M NaCl; (b) casein kinase peak emerged from P-cellulose column with washing buffer. Similar electrophoretic patterns were obtained when whole casein (0.5 mg) was replaced by $\beta$-casein (0.5 mg) as phosphorylatable substrate. Experimental conditions for acid hydrolysis and electrophoresis as previously described [1].

with washing buffer, phosphorylates both serine and threonine residues of whole casein and $\beta$-casein (Fig. 4b), indicating that it contains a casein kinase (referred to as 'casein kinase TS') which differs from casein kinase S in catalytic properties.

In conclusion, the above results show that the casein kinase activity of human erythrocyte hemolysate is due to two different enzyme forms (designated S and TS), which can be resolved and completely recovered by DEAE and subsequent P-cellulose chromatography in the presence of 0.2% Triton X-100, while they undergo a drastic and rapid loss of activity when the chromatographic procedures are carried out with buffered solutions which do not contain Triton.

Such extreme instability may account for the very poor yield (4—22%) obtained in our and other laboratories [7—8] when the casein kinases were purified by usual chromatographic procedures in the absence of Triton.

At present, our interest is devoted to casein kinase S, since it has been found, unlike casein kinase TS, to markedly phosphorylate the crude membrane proteins [2] and, in particular, isolated spectrin (the major membrane protein) [3].

By the above purification procedure, cytosolic casein kinase S, besides being purified about 5000-fold, is completely freed from the histone kinase activity.

On the basis of physico-chemical and catalytic properties, casein kinase S of human erythrocytes closely resembles the casein kinase I, purified from rabbit reticulocytes [9], i.e., both casein kinases display quite similar chromatographic behaviour, the same optimal pH range and the same ability to phosphorylate preferentially serine residues of $\beta$-casein [10].

The casein kinase TS of human erythrocytes appear to resemble the casein kinase II of rabbit reticulocytes [9] since both enzymes are found to phosphorylate, in addition to serine residues, also threonine residues of casein [10].

## Acknowledgements

The authors wish to thank Ms. Carla Munari for skilfull technical assistance, Ms. Maurizia Cuccia for secretarial help and Mr. Giorgio Parpajola for drawing the graphs.

## References

1 Clari, G., Michielin, E. and Moret, V. (1978) Biochim. Biophys. Acta 539, 420—426
2 Michielin, E., Clari, G. and Moret, V. (1979) Int. J. Biochem. 10, 171—175
3 Clari, G. and Moret, V. (1981) Biochim. Biophys. Acta 640, 240—251
4 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
5 Wang, C.S. and Smith, R.L. (1975) Anal. Biochem. 63, 414—417
6 Makkar, H.P.S., Sharma, O.P. and Negi, S.S. (1980) Anal. Biochem. 104, 124—126
7 Tao, M., Conway, R. and Cheta, S. (1980) J. Biol. Chem. 255, 2563—2568
8 Boivin, P. and Galand, C. (1979) Biochem. Biophys. Res. Commun. 89, 7—16
9 Hathaway, G.M. and Traugh, J.A. (1979) J. Biol. Chem. 254, 762—768
10 Tuazon, P.T., Bingham, E.W. and Traugh, J.A. (1979) Eur. J. Biochem. 94, 497—504
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275