Biochimica et Biophysica Acta, 523 (1978) 469—476

BBA 68391

DIPEPTIDYL CARBOXYPEPTIDASE FROM HUMAN SEMINAL PLASMA

D. DEPIERRE [a], J.-P. BARGETZI [b] and M. ROTH [a]

[a] *Laboratoire central de chimie clinique, Hôpital cantonal, Genève and* [b] *Département de biochimie, Faculté des Sciences de l'Université, Genève (Switzerland)*

(Received September 5th, 1977)

Summary

Dipeptidyl carboxypeptidase (angiotensin I converting enzyme) was purified from human seminal plasma. The apparent relative molecular mass determined by gel filtration on Sephadex G-200 was 330 000. The p*I* in isoelectric focusing was 4.6—5.0 and the optimum pH 7.7—8.0. The enzyme is activated by chloride. These properties are similar to those reported for the lung enzyme. The specificity is that of a carboxypeptidase releasing dipeptides. A study of different substrates showed the activity to be highest with Z-Leu-Gly-Gly, followed by Z-Phe-His-Leu > bradykinin > Bz-Gly-Gly-Gly > Boc-Phe-Ala-Pro > Bz-Gly-His-Leu > angiotensin I.

Introduction

Skeggs et al. [1] have described a peptidase present in blood plasma which they have called "angiotensin converting enzyme". Lung contains high amounts of this enzyme [2,3], which releases the C-terminal dipeptide from different polypeptides such as angiotensin I and bradykinin [4,5]. The name dipeptidyl carboxypeptidase, which has been proposed for this enzyme [6], will be used in this paper.

Since high amounts of the enzyme also occur in semen [7], we were interested to know how the seminal enzyme, a product of external secretion, compares with the enzyme of lung [8,9] and blood plasma [10,11]. We present a purification of the enzyme from human seminal plasma and a study of some of its properties.

Materials and Methods

Chemicals. DEAE-cellulose SS and CM-cellulose were purchased from Serva, Heidelberg; the Sephadex products and Blue Dextran were from Pharmacia, Uppsala, Sweden. Calibration proteins for the determination of molecular mass

obtained from Boehringer, Mannheim, G.F.R., as Combithek, size II, were: bovine serum albumin (M_r = 67 000); rabbit muscle aldolase (M_r = 158 000); catalase (M_r = 240 000); and beef liver glutamate dehydrogenase (M_r = 336 000). Coomassie Brilliant Blue R 250 was from Merck, Darmstadt, G.F.R.

The substrates Z-Phe-His-Leu, Bz-Gly-His-Leu, Z-Gly-Pro-Leu-Ala-Pro as well as angiotensin II were from Bachem, Marina del Rey, Calif. U.S.A. Angiotensin I, bradykinin and Bz-Gly-Gly-Gly were obtained from Schwarz-Mann, Orangeburg (N.Y., U.S.A.), and Z-Leu-Gly-Gly from Fluka, CH-9740 Buchs, Switzerland. Boc-Phe-Ala-Pro was synthesized with the kind help of Dr. Jean-Luc Fauchère in the Institute for molecular biology and biophysics, Eidgenössische Technische Hochschule, Zurich. Rat testis specific basic protein was kindly supplied by Dr. W. Stephen Kistler of the University of Chicago.

Biological material. Human sperm was supplied by the Sterility Centre of our Hospital (Dr. J. Meylan). It was frozen at −18° C no later than 3 h after ejaculation and stored at this temperature.

Enzyme assays. Dipeptidyl carboxypeptidase was determined as described previously [12] by incubation for 15 min at 37° C, in phosphate/borate buffer (pH 8) with 33 μg/ml of the substrate, Z-Phe-His-Leu. For the study of the different substrates, samples were incubated for 15 h and 57 μl aliquots were then analyzed for dipeptide release in an amino acid analyzer by use of the fluorimetric phthaldehyde reaction and electronic integration [13]; zero-time blanks and incubations without enzyme were analyzed similarly. Results are expressed in units (U), 1 U corresponding to the conversion of 1 μmol substrate per min.

Aldolase and glutamate dehydrogenase were determined by measurement of absorbance of NADH at 340 nm, and catalase by measurement of H_2O_2 decomposition at 240 nm [14].

Other assays. Protein was determined at 280 nm with blank correction at 260 nm according to Warburg and Christian [15]. For very dilute solutions, a fluorimetric method [16] was used.

Purification of seminal dipeptidyl carboxypeptidase. Samples of frozen human sperm were brought to 4° C and pooled (total volume 910 ml). Subsequent operations were done at 4° C. The pool was centrifuged for 1 h at 20 000 × *g*; the clear supernatant seminal plasma was dialyzed three times against 10 vols. of 1 mmol/l potassium phosphate (pH 7). The supernatant obtained after centrifugation at 2500 × *g* was fractionated at 1.6—2.7 mol/l of $(NH_4)_2SO_4$. The precipitate was dissolved in buffer, dialyzed against phosphate (pH 7) and fractionated at 2.0—2.5 mol/l $(NH_4)_2SO_4$. The precipitate was dissolved in a small amount of potassium phosphate (pH 7), dialyzed once against the same buffer and three times against 10 mmol/l sodium acetate (pH 4.7). The solution was then submitted to batch adsorption on CM-cellulose at pH 4.7 to remove extraneous protein, dialyzed against potassium phosphate (pH 7), and passed through a 2.5 × 31 cm column of DEAE-cellulose equilibrated with potassium phosphate (pH 7). The enzyme, which was retained on top of the column, was eluted with an NaCl gradient from 0—0.2 mol/l. Those fractions displaying high enzyme activity were pooled and dialyzed three times against 10 mmol/l potassium phosphate (pH 7). The solution was submitted to a second chromatography on DEAE-cellulose. Those fractions eluted at 70—90 mmol/l of NaCl were pooled, concentrated, and dialyzed against three changes of 50 mmol/l

potassium phosphate (pH 7). The preparation was then passed through a column (1.5 × 79 cm) of Sephadex G-200 equilibrated with 50 mmol/l phosphate (pH 7). The enzyme emerged as a single peak which was concentrated and rechromatographed on Sephadex G-200 under the same conditions. The fractions of the active peak were pooled and concentrated to 4.6 ml, providing the purified enzyme preparation. The concentration steps were done with Sephadex G-25 Coarse [17].

Determination of the relative molecular mass. The enzyme solution was loaded onto a 1.5 × 79 cm column of Sephadex G-200 Superfine equilibrated with 50 mmol/l potassium phosphate (pH 7), together with solutions of albumin, aldolase, catalase and glutamate dehydrogenase. Elution was performed with the same buffer. The void volume was determined with blue dextran and the relative molecular masses (M_r) were calculated by use of the average partition coefficient K_{av} (ref. 18).

Gel electrophoresis. This was carried out according to Maurer [19] in 5 × 75 mm tubes with a gel containing 70 g/l polyacrylamide. Every sample was run in duplicate, yielding one gel for protein staining and the other for enzyme localization. Staining was done with Coomassie brilliant blue R 250 [20]. The unstained gel was sliced into 30 segments, 1.5 mm wide, and the separate slices were allowed to stand at 4°C for 24 h in 1 ml of 50 mmol/l potassium phosphate (pH 7). After mixing, 50 µl of the resulting solution were incubated for 2 h at 37°C with 50 µl substrate solution (Z-Phe-His-Leu, 20 mg/ml) and 3 ml of buffer, and histidyl-leucine was determined as described previously [12].

Isoelectric focusing. This was performed by the method of Conway-Jacobs [21] with a pH gradient from 4—6. From duplicate runs, one gel was stained by the technique of Malik and Berrie [22], whereas the other was sliced into segments 1.5 ml wide. The separate segments were put into tubes containing 1 ml water. After brief agitation, the pH was determined in the eluate; determination of enzyme activity was carried out as for gel electrophoresis.

Results

The purification from human semen was made with a procedure similar to that used by Dorer et al. [23] for the hog lung enzyme. The yields of the different steps are shown in Table I. Dialysis of the crude seminal plasma against phosphate buffer consistently results in an increase of the measured enzyme activity; this may be due to the removal of dialyzable inhibiting substances. The overall purification factor relative to the starting material was 430 times, with a final yield of 0.7%. The final preparation still showed two bands on gel electrophoresis; that band displaying enzymatic activity has an electrophoretic mobility at pH 8.9 of 0.29 relative to bromophenol blue (Fig. 1).

Relative molecular mass of the enzyme. Estimation of the relative molecular mass (M_r) by gel filtration on Sephadex G-200 yielded a K_{av} of 0.036 and an M_r of 330 000.

Isoelectric point. After isoelectric focusing of 250 µg enzyme preparation from the first DEAE-cellulose chromatography, enzyme activity was found to be located in those fractions of pH 4.6—5.0. This may be compared to the values of 4.3 and 5.2 reported for the hog lung and kidney enzymes respectively [24,25], and of 4.75 for rabbit lung [26].

TABLE I

PURIFICATION OF HUMAN SEMINAL DIPEPTIDYL CARBOXYPEPTIDASE

No.	Step	Volume (ml)	Protein (mg/ml)	Enzyme activity		Yield (%)	Purification factor
				U	U per g protein		
1	Seminal plasma	910	12.3	19.2	1.7	100	1.0
2	Dialyzed seminal plasma	1020	10.5	27.3	2.5	142	1.5
3	Ammonium sulfate fractionation 1.6—2.7 mol/l	170	25.9	11.8	2.7	61	1.6
4	CM-cellulose batch pH 4.7	47.3	2.8	1.33	10.0	6.9	5.9
5	DEAE-cellulose No. 1	4.3	12.1	0.86	16.5	4.4	9.7
6	DEAE-cellulose No. 2	5.5	2.9	0.835	52.0	4.3	30.6
7	Sephadex G-200 No. 1	6.0	0.12	0.169	234.0	0.9	137.6
8	Sephadex G-200 No. 2	4.6	0.04	0.135	730.0	0.7	429.4

Influence of pH. Fig. 2 shows the activities of crude undialyzed seminal plasma and of a purified sample of seminal dipeptidyl carboxypeptidase resulting from the second DEAE-cellulose chromatography. In both cases the optimum pH was 7.7—8.0.

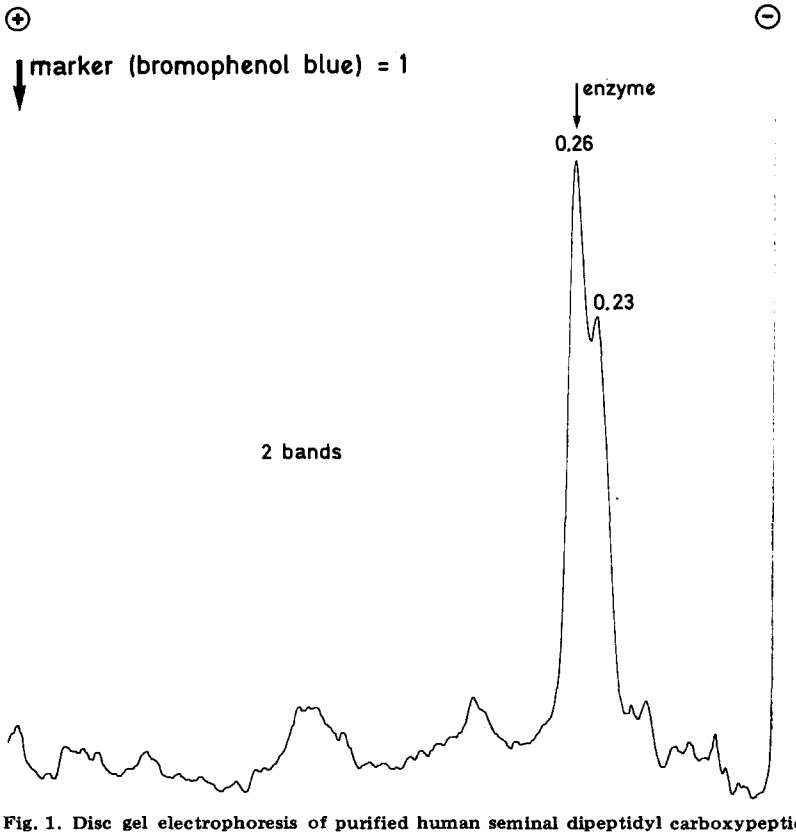

Fig. 1. Disc gel electrophoresis of purified human seminal dipeptidyl carboxypeptidase. Electrophoresis was carried out as under Materials and Methods. The figure represents the stained gel.

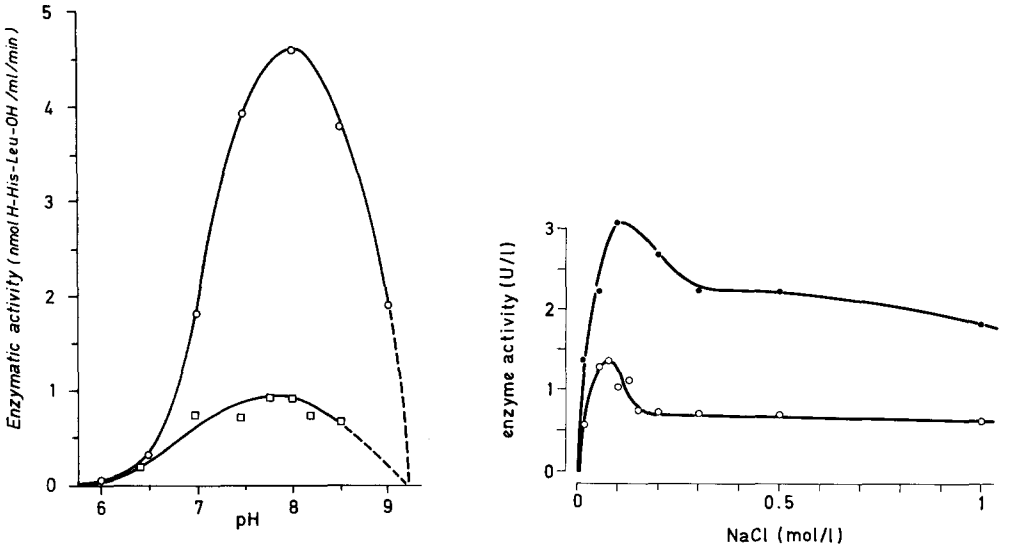

Fig. 2. Effect of pH on the relative enzyme activity of seminal dipeptidyl carboxypeptidase. □——□, crude, non-dialyzed human seminal plasma. ○——○, partially purified human seminal plasma (active fraction from step 6). Enzyme was assayed as under Materials and Methods.

Fig. 3. Influence of chloride concentration on the enzymatic activity of preparations of human seminal plasma. Increasing amounts of NaCl were incorporated in the incubation medium as described under Materials and Methods. Closed circles, crude human seminal plasma. Open circles, purified enzyme (step 6).

Influence of chloride and of EDTA. The effect of addition of NaCl to either non-dialyzed or dialyzed seminal plasma is shown in Table II. An enhancement of activity is observed. It is stronger for dialyzed samples, probably because of the higher chloride content of non-dialyzed samples. Thus, the same type of activation is observed for the seminal enzyme as for the blood plasma and lung enzyme [27]. That the effect is due to the chloride and not to the sodium ion has been demonstrated earlier for the blood plasma enzyme [28].

If the chloride-activated samples are incubated in presence of 1 mmol/l of EDTA, an inhibition of similar magnitude is observed both for dialyzed and undialyzed samples. The influence of chloride concentration is shown in Fig. 3. Maximum activity is observed at 75 mmol/l NaCl both for the crude and the purified enzyme.

Stability. Whereas the enzyme of crude seminal plasma is stable when incubated for 15 min below 40° C, the same treatment at 60° C produces a 75% loss of activity, and at 70° C a complete inactivation.

The enzyme is rapidly inactivated at pH 4.0, and shows the highest stability from pH 7—10.

Specificity. Highly purified seminal dipeptidyl carboxypeptidase resulting from the second gel filtration on Sephadex G-200 was incubated with different peptides. Table III shows that the enzyme is capable of releasing the C-terminal dipeptide from polypeptide substrates of various C-terminal structure. From the comparison between Bz-Gly-Gly-Gly and Bz-Gly-His-Leu, it appears that a C-terminal glycyl-glycine is more susceptible to enzyme action than is histidyl-

TABLE II

INFLUENCE OF CHLORIDE ION AND OF EDTA ON HUMAN SEMINAL DIPEPTIDYL CARBOXYPEPTIDASE

Sperm No.		Spermatozoa count (10^6/ml)	Enzyme activity (U/l)		
			Without Cl^-	With 200 mmol/l Cl^-	With 200 mmol/l Cl^- plus 1 mmol/l EDTA
1	Non-dialyzed	202	0.8	5.5	1.7
2	Non-dialyzed	1	0.5	1.9	0.5
3	Non-dialyzed	15	0.6	2.2	0.6
4	Non-dialyzed	82	0.6	2.6	0.7
5	Dialyzed	26	1.7	15.5	3.0
6	Dialyzed	2	1.0	27.2	4.9
7	Dialyzed	46	1.1	22.9	4.0
8	Dialyzed	60	1.0	26.7	5.7

leucine. The order in the rate of hydrolysis of the three substrates Z-Phe-His-Leu > angiotensin I ⩾ Bz-Gly-His-Leu was not essentially different when these were incubated with preparations of the different steps of the purification procedure. A similar relationship between the activities towards Z-Phe-His-Leu and angiotensin I has been observed with the enzyme from human blood serum [28] and from calf lung [29].

The preparation of rat testis specific basic protein [30] did not yield measurable quantities of C-terminal His-Leu upon incubation.

Origin of the enzyme. We did not investigate the question of the origin of the enzyme. However, two of the seminal plasma samples mentioned in Table II were azospermic, and it can be seen that these had an enzyme activity similar to other samples rich in spermatozoa. Thus, dipeptidyl carboxypeptidase does not originate from spermatozoa.

TABLE III

DIPEPTIDYL CARBOXYPEPTIDASE ACTIVITY WITH DIFFERENT SUBSTRATES

Incubations were carried out for 15 h at 37°C with 2 μl of enzyme solution in a total volume of 55 μl except for bradykinin where the total volume was 95 μl. Other conditions as under Materials and Methods.

Substrate	Substrate concentration (mmol/l)	Activity of enzyme solution (U/l)	Product measured
Z-Phe-His-Leu	3.3	6.7	His-Leu
Bz-Gly-His-Leu (hippuryl-His-Leu)	33.0	0.6	His-Leu
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (Angiotensin I)	33.0	0.5	His-Leu
Rat testis-specific basic protein (Ser-His^{40}-Leu^{41})	0.4	<0.2	His-Leu
Z-Leu-Gly-Gly	3.3	11.5	Gly-Gly
Bz-Gly-Gly-Gly (hippuryl-Gly-Gly)	3.3	2.2	Gly-Gly
Boc-Phe-Ala-Pro	3.3	0.7	Ala-Pro
Z-Gly-Pro-Leu-Ala-Pro	3.3	0.4	Ala-Pro
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin)	4.8	2.9	Phe-Arg

Discussion

Several properties of the seminal dipeptidyl carboxypeptidase are similar to those of the lung enzyme: (i) activation by chloride; (ii) inhibition by EDTA, and, as we shall report, by the nonapeptide <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (SQ 20881) (Depierre, Bargetzi and Roth, unpublished); (iii) pH optimum between 7.7 and 8.0; (iv) activity against the natural substrates angiotensin I and bradykinin, and (v) against several artificial substrates.

The apparent molecular weight of 330 000 is consistent with the value of 300 000 observed by Dorer et al. [23] and Stevens et al. [29] for the pulmonary enzyme from hog and calf, respectively. The "true" molecular weight is likely to be much smaller, as it is for the lung and blood plasma enzyme when measured by gradient centrifugation [8,31]; it has been postulated that the higher values obtained by gel filtration are due to the oligosaccharide moiety of the enzyme [26,31].

Our studies with synthetic substrates demonstrate that the seminal enzyme, like the lung enzyme, has the specificity of a carboxypeptidase releasing the C-terminal dipeptide from a peptide chain. In our liquid chromatography system, the dipeptide corresponding to any given substrate was well separated from its constituent amino acids, and the chromatograms showed that dipeptide, and no single amino acid, was released.

The male reproductive tract secretes a number of enzymes, some of which, such as prostatic phosphatase, are exclusively produced in it. Others, however, are also synthesized in other tissues. Thus, two seminal pepsinogen fractions are identical with the group II pepsinogens of the stomach [32], despite the fact that their function in the seminal fluid may be quite different from that in the digestive tract. The same situation may well occur with dipeptidyl carboxypeptidase, the seminal and pulmonary forms of which are very similar. It is now certain that one role of the pulmonary enzyme is to transform circulating angiotensin I into the highly vasopressive angiotensin II. A similar mechanism is unlikely to take place in the seminal fluid itself, since this does not contain angiotensin II (Banichahi, F., unpublished data). However, it is interesting to note that the uterus contains renin, the enzyme leading to the production of angiotensin I. It is tempting to speculate that the fertilization process involves the enzymic production of angiotensin II within the uterine cavity. The fact that our enzyme preparation was capable of catalyzing the conversion of angiotensin I into angiotensin II is in favor of this hypothesis.

The data also show that bradykinin is a substrate. Whether this corresponds to another function of the seminal enzyme cannot be answered at present. To our knowledge, bradykinin has not been shown to occur in sperm, but on the other hand the uterus is one of the most sensitive organs to stimulation by bradykinin, and the fertilization process might perhaps involve bradykinin inactivation.

Acknowledgements

This work was supported by the Swiss National Science Foundation (grant No. 3.913.72).

We thank Dr. F. Banichahi, of the Division of Endocrinology in our Hospital, who investigated by radioimmunoassay whether angiotensin was present in seminal fluid.

References

1 Skeggs, L.T., Kahn, J.R. and Shumway, N.P. (1956) J. Exp. Med. 103, 295—299
2 Ng, K.K.F. and Vane, J.R., (1976) Nature 216, 762—766
3 Roth, M., Weitzman, A.F. and Piquilloud, Y. (1969) Experientia 25, 1247
4 Elisseeva, Y.E., Orekhovich, V.N., Pavlikhina, L.V. and Alexeenko, L.P. (1971) Clin. Chim. Acta 31, 413—419
5 Yang, H.Y.T., Erdös, E.G. and Levin, Y. (1971) J. Pharmacol. Exptl. Therap. 177, 291—300
6 Yang, H.Y.T., Erdös, E.G., Jenssen, T.A. and Levin, Y. (1970) Fed. Proc. 29A, 281
7 Cushman, D.W. and Cheung, H.S. (1971) Biochim. Biophys. Acta 250, 261—265
8 Fitz, A. and Overturf, M. (1972) J. Biol. Chem. 247, 581—588
9 Boaz, D., Wyatt, S. and Fitz, A. (1974) Fed. Proc. 33, 1234
10 Lee, H.J., Larue, J.N. and Wilson, I.B. (1971) Arch. Biochem. Biophys. 142, 548—551
11 Fitz, A., Boyd, G.W. and Peart, W.S. (1971) Circ. Res. 28, 246—253
12 Depierre, D. and Roth, M. (1975) Enzyme 19, 65—70
13 Roth, M. (1976) J. Clin. Chem. Clin. Biochem. 14, 361—364
14 Aebi, H. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H.U., ed.), 3rd edn., pp. 713—724, Verlag Chemie, Weinheim
15 Warburg, O. and Christian, W. (1942) Biochem. Z. 310, 384—421
16 Weidekamm, E., Wallach, D.F.H. and Fluckiger, R. (1973) Anal. Biochem. 54, 102—114
17 Flodin, P., Gelotte, B. and Porath, J. (1960) Nature 188, 493
18 Andrews, P. (1965) Biochem. J. 96, 595—606
19 Maurer, H.R. (1968) Disk-Elektrophorese, pp. 42—45, W. de Gruyter, Berlin
20 Chrambach, A., Reisfeld, R.A., Wyckoff, M. and Zaccari, J. (1967) Anal. Biochem. 20, 150—154
21 Conway-Jacobs, A. and Lewin, L.M. (1971) Anal. Biochem. 43, 394—400
22 Malik, N. and Berrie, A. (1972) Anal. Biochem. 49, 173—176
23 Dorer, F.E., Kahn, J.R., Lentz, K.E., Levine, M. and Skeggs, L.T. (1972) Circ. Res. 31, 356—366
24 Nakajima, T., Oshima, G., Yeh, H.S.J., Igic, R. and Erdös, E.G. (1973) Biochim. Biophys. Acta 315, 430—438
25 Oshima, G., Gecse, A. and Erdös, E.G. (1974) Biochim. Biophys. Acta 350, 26—37
26 Soffer, R.L. (1976) Ann. Rev. Biochem. 45, 73—94
27 Bakhle, Y.S. (1973) in Handbook of Experimental Pharmacology, Vol. 37 (Page, I.H. and Bumpus, F.M., eds.) p. 58, Springer, Berlin
28 Piquilloud, Y., Reinharz, A. and Roth, M. (1970) Biochim. Biophys. Acta 206, 136—142
29 Stevens, R.L., Micalizzi, E.R., Fessler, D.C. and Pals, D.T. (1972) Biochemistry 11, 2999—3007
30 Kistler, W.S., Noyes, C., Hsu, R. and Heinrikson, R.L. (1975) J. Biol. Chem. 250, 1874
31 Das, M. and Soffer, R.L. (1975) J. Biol. Chem. 250, 6762—6768
32 Samloff, I.M. and Liebmann, W.M. (1972) Clin. Exptl. Immunol. 11, 405—414