[CONTRIBUTION FROM THE PROTEIN SECTION, ARMOUR LABORATORIES, RESEARCH DEPARTMENT]

Partial Purification and Characterization of Pankrin, a New Pancreatic Proteinase

BY NORMAN H. GRANT AND KENNETH C. ROBBINS

RECEIVED JUNE 22, 1956

The partial purification of a water-insoluble proteinase, pankrin, is described. The enzyme is prepared from a water extract of pancreatin powder by adsorption on Amberlite IRC-50 (XE 97) at *p*H 4.7 at low ionic strength and elution at the same *p*H at high ionic strength. The enzyme is characterized by its water insolubility and by its solubility properties at low ionic strength. It is differentiated from other pancreatic proteinases not only by its solubility properties but by its high activity on protein substrates and its low activity on the synthetic substrates TSAME and ATEE.

Introduction

The occurrence of a previously unrecognized pancreatic proteinase has been reported from this Laboratory.[1] The enzyme has been designated *pankrin* and exhibits the following characteristics which distinguish it from trypsin or chymotrypsin: (a) The hydrolytic activity against several protein substrates is high in comparison with the activities of trypsin and chymotrypsin, while the hydrolytic activity against specific synthetic substrates is low. (b) Pankrin is less sensitive than trypsin to pancreatic and soybean trypsin inhibitors and less sensitive than chymotrypsin to β-phenylpropionate. (c) Pankrin, unlike trypsin, does not clot citrated plasma.

The present paper describes the methods which led to the separation of pankrin, and it reports a highly distinguishing characteristic of the enzyme—its water insolubility. Previous work[2] has shown the presence of water-insoluble proteinase(s) in pancreatic extracts, and experiments reported here confirm the existence of water-insoluble proteinase(s) in pancreatic juice.

Experimental

Assays.—Proteinase assays employing hemoglobin, serum albumin and casein were performed at 37° according to the method of Anson.[3] One proteinase unit was defined as the amount of enzyme activity capable of causing the liberation of 1 milligram of tyrosine from the substrate in 10 minutes. Hemoglobin was used in the assays accompanying the isolation. Esterase assays on *p*-toluenesulfonyl-L-arginine methyl ester (TSAME) were carried out by the method of Schwert and co-workers[4] and on acetyl-L-tyrosine ethyl ester (ATEE) by the method of Kaufman and co-workers.[5] Milk clotting was assayed by the method of Kunitz and Herriott.[6] Protein was determined by a biuret method[7,8] or by a Folin–Ciocalteau phenol method,[9] using crystalline bovine albumin as the standard.

Preparation.—To each kg. of pancreatin (dried, defatted porcine pancreas containing 8% NaCl and 10% porcine duodenum) was added 5 liters of distilled water, and the mixture was stirred at room temperature for 3–5 hours. The insoluble residue was removed by suction filtration using 4–8% Celite 535. To each volume of filtrate was added 2.5 volumes of 10% Rohm and Haas Amberlite IRC-50 (XE 97) cation-exchange resin (equilibrated with and suspended in 0.05 *M* sodium acetate–acetic acid buffer *p*H 4.7) and 5 volumes of distilled water, giving a final ionic strength of 0.05. The mixture was stirred for approximately 2 hours at room temperature. The resin was removed by centrifugation for 5 minutes at 1100 × *g*, and the supernatant solution was discarded. A precipitate which formed during the mixing, probably as a result of lowering the *p*H, was easily decanted. The resin was washed twice with 2.5 volumes of water for 15 minutes, and the washes were discarded. The enzyme was then eluted by stirring the resin for 2 hours with 1 volume of 2.0 *M* sodium acetate–acetic acid or ammonium acetate–acetic acid buffer *p*H 4.7. Following centrifugation at 1100 × *g*, the eluate, E-1, was freed of traces of resin by filtration through Schleicher and Schuell 595 paper. The enzyme was further purified in several ways.

First, the adsorption on XE 97 and elution therefrom was repeated. One volume of E-1 was stirred at room temperature for 30 minutes with 7 volumes of water and 0.7 volume of packed, equilibrated XE 97 resin (0.05 *M* acetate buffer *p*H 4.7).[10] After centrifugation, the enzyme was eluted from the resin by 1.2 volumes of 2.0 *M* acetate buffer *p*H 4.7, giving eluate E-2.[11]

In another procedure, the pankrin in E-1 was precipitated by adding 95% ethanol at −5° to a final concentration of 80%. Below this concentration, negligible precipitation occurred. Alternatively, the pankrin was precipitated by full saturation with ammonium sulfate at 1°. The ethanol precipitate, P-1, and the ammonium sulfate precipitate, P-1a, were both dried by lyophilization. The enzymatic activities of the above fractions are summarized in Table I.

Solubility Studies.—P-1 was only partially soluble in water. A single water extraction of 2% solids at 1° resulted in solubilization of 17–27% of the proteinase activity. After 2 water extractions, the water-insoluble residue, P-1r, contained 45–62% of the activity and showed greater activity than P-1 on hemoglobin (27%), ATEE (54%), and in milk clotting (33%).

P-1a was dissolved in distilled water and was dialyzed at 1° against 20 volumes of distilled water with several changes. The small precipitate that formed was removed by centrifugation at 3100 × *g* and 1° for 1 hour and discarded. The supernatant solution was lyophilized and the solid was redissolved at room temperature to give a 2% aqueous solution. An equal volume of 4 *M* ammonium sulfate was added, and the mixture was allowed to stand for 24 hours at room temperature. The precipitate was collected by centrifugation at room temperature, was redissolved in water and was dialyzed first against running tap water at 2° overnight and finally against cold distilled water. A precipitate formed and was separated by centrifugation. The water-soluble and water-insoluble fractions were lyophilized and designated P-2 and P-3, respectively. The enzymatic activities of P-1r, P-2, and P-3 are summarized in Table I. A repeated dialysis of P-2, dissolved

(1) N. H. Grant and K. C. Robbins, THIS JOURNAL, **77**, 2027 (1955).

(2) N. H. Grant and K. C. Robbins, to be published.

(3) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

(4) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).

(5) S. Kaufman, H. Neurath and G. W. Schwert, *ibid.*, **177**, 793 (1949).

(6) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia Univ. Press, New York, N. Y., 1948, p. 303.

(7) H. W. Robinson and C. G. Hogden, *J. Biol. Chem.*, **135**, 707 (1940).

(8) H. W. Robinson and C. G. Hogden, *ibid.*, **135**, 727 (1940).

(9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *ibid.*, **193**, 265 (1951).

(10) One g. of dry resin occupies 2.75 ml. when wet and packed.

(11) The preparation described in the preliminary report[1] was an eluate E-2. Since it has not been possible to obtain this fraction in the dry state free of salt without losing most of the activity, the specific activity has been expressed in terms of protein. The protein values (Folin–Ciocalteau phenol method) for the preparation before and after dialysis agree closely. The crystalline trypsin and chymotrypsin preparations, used for comparisons, were composed entirely of protein, as determined by the quantitative biuret or Folin–Ciocalteau methods.

TABLE I

ENZYMATIC ACTIVITIES AGAINST DIFFERENT SUBSTRATES

Fraction no.	Preparation	Hemoglobin	Serum albumin	Casein	ATEE	TSAME
	Tissue extract	1.1[a]				
E-1	First eluate	6.4–8.7[a]				
E-2	Second eluate	37.6–38.4[a]	6.2[a]	31.6[a]	0.187[a]	0.138[a]
P-1	Ethanol ppt.	6.5–7.5	1.2	6.4	.032–0.037	.000–0.002
P-1a	Ammonium sulfate ppt.	6.9–9.2[a]			.034[a]	.021[a]
P-1r	Water-washed P-1	9.0–10.0			.057	
P-2	Dialysis supernatant	9.6			.059	.012
P-3	Dialysis precipitate	14.8			.042	.006
	Trypsin	11.6	4.8	11.5	.012	.403
	Trypsin	19.5	5.1	17.2	.003	.478
	α-Chymotrypsin	5.7	2.4	18.4	.396	.000
	α-Chymotrypsin	6.7	0.6	12.8	.370	.001

[a] Specific activity is expressed in units per mg. of protein. All other values are units per mg. of solid.

in water, led to another precipitation; dialysis of the soluble fraction for this precipitation led to yet another partition between soluble and insoluble components. The specific proteinase activities of the soluble components resulting from the 3 successive dialyses were 9.6, 7.8 and 8.1 units per mg. The corresponding activities of the insoluble components were 14.8, 12.0 and 10.9 units per mg.

The solubility behavior of P-3 as a function of *p*H was studied by making up 0.1% suspensions in acetate, Sørensen phosphate, and Sørensen borate buffers at ionic strength 0.01 and allowing them to stand at 1° for 90 minutes with frequent stirring. The suspensions were centrifuged at 1100 × *g* and 4° for 30 minutes. Determination of *p*H, proteinase activity and optical density at 280 mμ were made on the supernatant solutions. The protein was completely soluble at *p*H 3.5, providing a baseline for comparisons (Fig. 1). As the *p*H was increased to approximately 6, the solubility of both total solids and proteinase fell. Between 6.0 and 7.5 both solubilities were relatively constant. From *p*H 7.5 to *p*H 8.5 the solubility of the solid rose, while that of the proteinase remained essentially level.

To determine whether the divergence in the two solubilities at the higher *p*H's was real, and not a reflection of enzyme instability, the enzyme (P-3) was extracted in a concentration of 1 mg. solids per ml. at ionic strength 0.15 and *p*H's 3.52, 7.28 and 9.32 for 90 minutes at 1°. It was expected that at this ionic strength total solution would occur and stability at the 3 *p*H's could be compared. Complete solution occurred at *p*H 7.28 and *p*H 9.32, and the specific activities (14.2 and 14.5) were not changed. However, at *p*H 3.52 the solid did not completely dissolve and only 72% of the activity was extracted. This finding was extended by carrying out extractions of P-3 in *p*H 3.5 acetate buffers of ionic strength 0.01, 0.07, 0.15 and 0.20 under the above conditions. The solubilities of both the solid and proteinase activity decreased with increasing ionic strength and were only 60% of maximum at ionic strength 0.20. It is apparent, then, that in the pankrin-containing preparation P-3 the variation of proteinase solubility with ionic strength is a function of *p*H.

The foregoing data indicated the occurrence in the pancreas of a water-insoluble endopeptidase. This led to the question of whether such an enzyme also occurred in pancreatic juice. Dog pancreatic juice (6.4 ml., *p*H 8.5) was neutralized with HCl to *p*H 5.37. A heavy gelatinous precipitate formed and was removed by centrifugation at 4°. The supernatant solution was then dialyzed against 6 liters of distilled water for 36 hours with one change. The precipitate was removed by centrifugation at 4° and washed twice with 5-ml. volumes of distilled water. The washed precipitate and the supernatant solution from the dialysis were lyophilized and assayed. Preliminary treatment with 0.002% trypsin at *p*H 7.4 had no significant effect on the activity of either fraction; since the juice did not contain active trypsin following the collection, activation of zymogens probably occurred during the dialysis step. The specific proteinase activities were 8.7 units per mg. for the soluble fraction and 9.8 units per mg. for the insoluble fraction. The corresponding enzyme yields were 260 units and 244 units.

Discussion

In early studies of pancreatic enzymes,[12] discrepancies between proteolytic and milk clotting activities pointed to the existence of at least two pancreatic endopeptidases. Our belief that there was a third endopeptidase was at first based upon very similar grounds—marked differences in ability to attack known substrates. To this evidence can now be added differentiation by solubility behavior. Pankrin is a water-insoluble proteinase, while trypsin and chymotrypsin are water soluble.

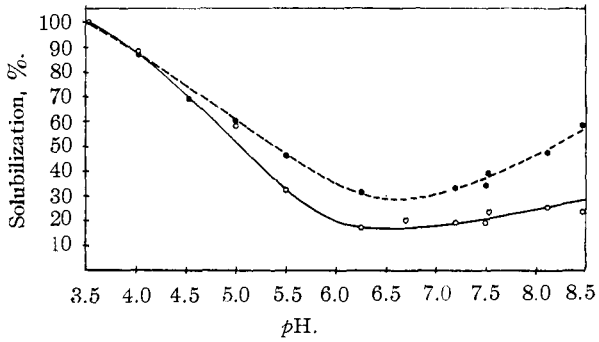

Fig. 1.—Solubility of pankrin fraction P-3 as a function of *p*H at ionic strength 0.01: , solid; O, proteinase activity (denatured hemoglobin).

Dialysis of pancreatin extracts and pancreatic juice results in the formation of a protein precipitate with high proteolytic activity. Pankrin is probably a part of this mixture of proteolytic enzymes, which includes other activities such as elastase and carboxypeptidase. After the substrate specificity is defined, pankrin can be purified from this complex.

The solubility properties of pankrin fractions P-2 and P-3 suggest the reversibly dissociable component systems of serum globulins described by Sørensen.[13,14] The precipitate insoluble in 2 *M* ammonium sulfate gave on dialysis the soluble component, P-2, and the insoluble component, P-3. Removal of P-3 in effect shifted the equilibrium,

(12) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia Univ. Press, New York, N. Y., 1948, pp. 96–97.

(13) S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 11 (1925).

(14) S. P. L. Sørensen, *ibid.*, **18**, No. 5 (1930).

and upon further dialysis of P-2 a second insoluble component separated. Repeating this process gave a third partition. The solubility of the dissociable component system at a given stage was a reflection of the relative amounts of soluble and insoluble proteins. Thus, the ratio of soluble to insoluble solids after the dialysis of P-2 was 1.2, while this ratio after the subsequent dialysis of the soluble component was 5.1.

It may be noted from Table I that the ratio of activity on hemoglobin to activity on ATEE is between 158 and 352 for pankrin fractions and between 14 and 18 for the two crystalline α-chymotrypsin preparations. The ratios of activity on hemoglobin to activity on TSAME are greater than 273 for pankrin and 29 and 41 for the two crystalline trypsin preparations. The very large spread in hemoglobin: TSAME for pankrin preparations indicates residual contamination with trypsin. The ATEE activities in pankrin may be the result of chymotrypsin contamination, but an inherent overlapping of specificities cannot be ruled out now. Fraction P-1r has 80% of the milk clotting activity of crystalline α-chymotrypsin but only 14% of the ATEE activity.

A preliminary digestion of corticotropin-A by fraction E-2 resulted in extensive cleavage of the molecule, as indicated by the appearance of at least 10 ninhydrin-positive spots on chromatograms of the digest.[15] Crystalline trypsin[16] and crystalline chymotrypsin[17-19] in similar experiments produced fewer cleavages of the peptide, indicating broader specificity for pankrin.

(15) W. F. White, The Armour Laboratories, personal communication.

(16) W. F. White and W. A. Landmann, THIS JOURNAL **77**, 1711 (1955).

(17) W. F. White and W. A. Landmann, *ibid.*, **76**, 4193 (1954).

(18) W. F. White, *ibid.*, **76**, 4194 (1954).

(19) W. F. White and W. A. Landmann, *ibid.*, **77**, 771 (1955).

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF ILLINOIS]

Pattern of Action of Crystalline Muscle Phosphorylase on Glycogen as Determined from Molecular Size Distribution Studies

BY J. LARNER, B. ROGER RAY AND H. F. CRANDALL

RECEIVED MARCH 26, 1956

The results of the action of crystalline muscle phosphorylase on rabbit muscle and liver glycogens have been studied. The sedimentation velocity diagrams from the ultracentrifuge have been analyzed according to the Baldwin–Williams procedure to yield distribution functions of the sedimentation coefficients; sedimentation and diffusion measurements on several fractions permitted transposition into molecular weight distributions. Liver glycogen showed a highly skewed distribution with molecular weights ranging from well below a million to over 100 million and with a broad maximum around 2 million. Muscle glycogen was less heterogeneous, containing species from several thousand up to 15 million and with a maximum around 2.5 million. Enzymatic degradations, carried out to various extents and under different pH conditions, showed that the highest molecular weight material most noticeably disappeared. The same general behavior was observed in every case. That is, a less heterogeneous sample resulted which had an increased proportion centered about approximately the same maximum as before degradation. Equilibration of glycogen and glucose 1-phosphate with phosphorylase shifted the molecular weight distributions in the same general manner as did degradation. The experimental results are consistent with the idea of greater probability of enzymatic action on the outer glucose chains of the largest molecules.

Introduction

The mechanism of action of crystalline muscle phosphorylase on the outer chains of branched polysaccharides of the glycogen–amylopectin class has been examined.[1] Analysis of the number of degraded outer chains susceptible to amylo-1,6-glucosidase action (units of limit dextrin configuration) during phosphorolysis has indicated that phosphorylase does not degrade by a "single chain" mechanism. It was also noted that the high yield of units of limit dextrin configuration from liver glycogen samples could be reduced by prior incubation (equilibration) of the glycogen with small amounts of glucose 1-phosphate and phosphorylase. This latter experiment was interpreted as indicating a redistribution or randomization of the glucose residues of the outer chains to produce a more uniform size distribution. In order to decide whether such a process could occur by an intermolecular mechanism, and also to determine whether phosphorolytic degradation had any degree of specificity with regard to molecular size of substrate, the partially degraded and the randomized glycogen samples have been examined ultracentrifugally. Sedimentation analyses have led to molecular weight distributions which indicate that in both cases the high molecular weight fraction of the polysaccharide is the preferential enzymatic substrate.

Experimental Details

Glycogen Samples.—Isolation of the glycogen samples both before and after enzymatic treatment has been discussed.[1,2] The samples studied by ultracentrifugal analysis are listed in Table I. With one exception, samples were reisolated after incubation with the enzyme by repeated ethanol precipitations after 1–2 minute treatment in 10–15% NaOH at 100°. The 21% degraded 117-1 was reisolated omitting the brief treatment with alkali.

Subfractions of glycogen samples were prepared by progressive addition of ethanol at 0° to solutions containing 2% glycogen and 2% NaCl. Precipitates were separated by centrifugation at 3°. They were dissolved in small volumes of water and dialyzed extensively against water at 3° to remove NaCl. Visually, fractions precipitating at lower ethanol concentrations were strikingly more opalescent than fractions precipitating at higher ethanol concentrations. Table II shows the ethanol concentrations used and the percentage distribution of the fractions. Recovery was about 99% in each case.

(1) J. Larner, *J. Biol. Chem.*, **212**, 9 (1955)

(2) B. Illingworth, J. Larner and G. T. Cori, *ibid.*, **199**, 631 (1952).