subunits do not involve charged groups. The interaction between subunits may be an entropy-driven process involving interaction between hydrophobic residues. It remains to be determined whether the four tyrosine groups per monomer which are exposed to water in the refolded monomer but masked in the native dimer are points of contact between the two subunits or are buried by a rearrangement which takes place during dimerization.

References

Linderstrøm-Lang, K. (1924), *Compt. Rend. Trav. Lab. Carlsberg 15*, 7.

Reynolds, J. A., and Schlesinger, M. J. (1967), *Biochemistry 6*, 3552.

Rothman, F., and Byrne, R. (1963), *J. Mol. Biol. 6*, 330.

Schellman, C., and Schellman, J. (1962), *Proteins 2*, 1.

Schlesinger, M. J. (1965a), *J. Biol. Chem. 240*, 4293.

Schlesinger, M. J. (1965b), *J. Biol. Chem. 240*, 4284.

Tait, G. H., and Vallee, B. L. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1247.

Tanford, C. (1955), *J. Phys. Chem. 59*, 798.

Tanford, C. (1957), *J. Am. Chem. Soc. 79*, 5340.

Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.

Tanford, C. (1967), *VII Intern. Congr. Biochem., Tokyo*, 5.

Tanford, C., and Epstein, J. (1954), *J. Am. Chem. Soc. 76*, 2163, 2170.

Tanford, C., and Kirkwood, J. G. (1957), *J. Am. Chem. Soc. 79*, 5333.

Preparation of Leucine Aminopeptidase Free of Endopeptidase Activity*

S. Ralph Himmelhoch and Elbert A. Peterson

ABSTRACT: A new method is described for preparing leucine aminopeptidase from the supernatant fraction of hog kidney which provides this enzyme in 50% yield with a specific activity (C') greater than 100 min^{-1}/mg of nitrogen, a value higher than that previously reported on a preparative scale. The purified enzyme did not detectably hydrolyze cytochrome *c*, rabbit actin, or mercuripapain, proteins whose amino-terminal sequences are refractory to exopeptidase action. The present preparation is therefore believed to be suitable for protein sequence determination. Previously unrecognized factors affecting the reproducibility of the preparation, including a requirement that kidneys from inbred hogs be employed, are discussed. Multiple chromatographic forms of the enzyme of differing substrate specificity were partially resolved.

The leucine aminopeptidase of the supernatant fraction of swine kidney can hydrolyze most L-amino acids from the amino-terminal position of proteins and peptides (Smith *et al.*, 1952). Exploitation of this property for protein sequence determination, however, requires enzyme preparations free of contaminating endopeptidases, because such contamination leads to erroneous deductions of sequence (Frater *et al.*, 1965).

Since available methods for preparing the enzyme (Frater *et al.*, 1965; Hill *et al.*, 1958; Fasold *et al.*, 1962; Folk *et al.*, 1959) do not eliminate endopeptidases, we have investigated alternative means of purifying it and have established a relatively rapid and reliable procedure for obtaining highly purified leucine aminopeptidase free of measurable endopeptidase activity. The enzyme prepared by this method has a molecular weight of 300,000 as determined by Sephadex G-200 chromatography, in agreement with the value deduced by earlier workers from its sedimentation constant (Spackman *et al.*, 1955). Its substrate specificity, pH optimum, and activation by Mn^{2+} are also in agreement with previous reports (Smith and Spackman, 1955). However, multiple chromatographic forms of the enzyme have been detected.

The physical and enzymatic properties of swine leucine aminopeptidase reported in this study and observed originally by Smith and Hill (1960) closely resemble the properties of the crystalline leucine aminopeptidase isolated from beef lens by Hanson *et al.* (1965). The crystalline enzyme from beef lens was found to have approximately the same molecular weight, substrate specificity, specific activity, pH optimum, and metal dependence as the enzyme obtained from swine kidney supernatant fraction.

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Public Health Service, Bethesda, Maryland 20014. *Received January 15, 1968.*

Methods

Assay of Enzyme Activity. Enzyme activity was measured by following the rate of liberation of leucine from leucinamide. Before carrying out the reaction, 1–5-μl aliquots of the samples to be tested were activated (Smith and Spackman, 1955) by incubation at 40° for 1 hr in 0.05 ml of a solution containing 0.001 M $MnCl_2$ and 0.1 M Tris-HCl buffer (pH 8.6). The reaction was started by adding 0.2 ml of a solution, warmed to 40°, containing 0.0625 M L-leucinamide, 0.001 M $MnCl_2$, and 0.1 M Tris-HCl buffer (pH 8.6). The reaction was stopped by adding to the reaction mixture, or to a suitable aliquot, an equal volume of 10% trichloroacetic acid.

Aliquots (5 μl) of the acidified reaction mixtures were spotted at 1-cm intervals along lines drawn 10 cm apart and perpendicular to the long dimension of a 46 × 57 cm sheet of Whatman No. 3MM paper. A total of 100 samples could conveniently be accommodated on a single sheet. The paper was sprayed with pyridine acetate buffer (pyridine–acetic acid–water, 1:10:89, pH 3.6) and electrophoresis was carried out for 10 min at 2000 V and 180 mA in a Gilson Model D high-voltage electrophoresis apparatus. The sheet was then dried at 60° for 20 min, dipped in ninhydrin solution (0.5% ninhydrin in 90% acetone), and developed for exactly 20 min at 60°. The blue spots corresponding to leucine and leucinamide were eluted with 5 ml of 70% ethanol, and the absorbance of the resulting solutions at 570 mμ was determined in a Beckman Model DU spectrophotometer. The fraction of the substrate which had been hydrolyzed was calculated from the expression $H = (L - B)/(A + (L - B)$, where L is the absorbance at 570 mμ of the eluted leucine spot, A the absorbance at 570 mμ of the corresponding leucinamide spot, and B the absorbance at 570 mμ of the leucine spot of a sample from which the enzyme had been omitted. When replicate spots were applied to the paper, electrophoresed, and eluted, the standard error of the mean value for per cent hydrolysis was ±6%.

Calculation of the first-order rate constant and of specific activity (C') were performed according to Smith and Spackman (1955), substituting colorimetric protein determination (Lowry *et al.*, 1951) for nitrogen measurement, except in the final preparations, where direct comparison with previously reported results was desired. Although active samples were easily identified by visual inspection of the ninhydrin-stained sheet, quantitative analysis was essential for properly selecting fractions for subsequent purification.

Peptide substrates (L-leucylglycine, L-glycylleucine, D-leucylglycine (all from Nutritional Biochemical Corp.), *N*-acetylglycylleucine, and carbobenzoxyglycylglycylphenylalanine (Mann)) were used at a concentration of 0.001 M in the same incubation medium used with leucinamide by following the increase in ninhydrin color as digestion proceeded. Results were expressed in terms of moles of leucine equivalent per mole of peptide substrate. When L-leucine-*p*-nitroanilide was employed as a substrate, the procedure of Wachsmuth *et al.* (1966) was followed, except that the reaction was carried out at pH 7.8 instead of pH 7.2.

Preparation of Homogenates and Detergent Precipitation. Mongrel hog kidneys were obtained in 10-kg lots from the local slaughterhouse. Inbred Yorkshire, Hampshire, and Spotted Poland China hog kidneys were obtained from the Arbogast and Bastion Meat Packing Co., Allentown, Pa.[1] All purification procedures were carried out at 4°.

The desired quantity of kidney was thawed at 4° for approximately 18 hr and dissected free of connective tissue, fat, and medulla. It was homogenized in 3 ml of 0.04 M Tris–0.005 M succinic acid–0.001 M $MgCl_2$–0.25 M sucrose per g of kidney used with a Waring Blendor for 30 sec. To the continuously stirred homogenate was added 0.15 ml of 10% (w/v) hexadecyltrimethylammonium bromide (CETAB, Technical, Eastman Organic Chemical Co., no. T5650) per g of kidney used. The homogenate was then centrifuged for 30 min at 2300 rpm and 4° in the 4-l. swinging-bucket rotor of an International Model PR-2 centrifuge. A clear pink supernatant solution and a copious rubbery precipitate were obtained. The supernatant solution (henceforth designated CETAB supernatant) served as the starting material for further preparation of leucine aminopeptidase. The precipitate contained leucine aminopeptidase M, the aminopeptidase of the microsomal fraction (Pfleiderer and Celliers, 1963).

Ion-Exchange Adsorbents. Two types of DEAE-cellulose were used: Whatman DE-50 Floc, lot 603–607, 100–200 mesh, nominal capacity 1.0 mequiv/dry g; and Whatman DE-23 (lot number not given), fibrous type, fines reduced, catalog no. 24233, nominal capacity 1.0 mequiv/dry g. Both adsorbent types were prepared for use in the same fashion, except that DE-50 Floc, but not DE-23, was sieved (Peterson and Sober, 1962) before use. All lots were tested by methods previously described (Himmelhoch and Peterson, 1966). A weighed quantity of the dry adsorbent was allowed to sink into 1 N NaOH (about 500 ml/100 g), and, after gentle stirring, the slurry was allowed to stand for 1 hr at room temperature. It was then transferred to a Büchner funnel and washed successively with water, 1 N HCl, water, 95% ethanol, water, 1 N NaOH, and water until the filtrate was no longer alkaline to hydrion paper. The washed adsorbent cake was suspended in the appropriate starting buffer and a quantity of the concentrated acid component of this buffer was added to adjust the adsorbent to the proper pH. It was then washed with three volumes of starting buffer and suspended for packing.

Columns under 2 cm in diameter were packed under air pressure gradually increased to 15 psi. Larger columns were packed by pumping the stirred slurry (about three volumes of buffer to one volume of adsorbent) into the column at a constant flow rate induced by a Roll-Flex pump (Himmelhoch *et al.*, 1966). After packing, the columns were transferred to the

[1] The authors thank Mr. William Clements Zinck for his kind advice concerning available strains of inbred hogs and assistance in making them available.

TABLE I: Purification of Leucine Aminopeptidase from 4 kg of Yorkshire Swine Kidney.

Fraction		Vol (ml) Fraction	Protein (mg/fraction)[a]	Enzyme Units (min^{-1}/ fraction)[b]	Sp Act. (min^{-1}/ mg)[c]	Yield (%)
Step	Description					
1	CETAB supernatant solution	10,000	106,000	2,400	0.12	100
2	Ion-exchange filtrate	660	10,000	2,400	1.5	100
3	Ammonium sulfate precipitate	120	2,500	1,900	4.5	80
4	Sephadex fraction	240	200	2,000	61	84
5	Fractions from gradient chromatography	300	30	1,200	120[d]	50

[a] Protein determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. [b] An enzyme unit is defined as the amount of enzyme yielding a first-order rate constant of 1 min^{-1}, *i.e.*, when $D/t \log 100/(100 - H) = 1\ min^{-1}$, where D is the dilution factor of the enzyme in the assay medium, t is the reaction time in minutes, and H is the per cent of the substrate hydrolyzed in t minutes. [c] The specific activity is defined as the first-order rate constant per mg of enzyme nitrogen. For fractions I–IV the enzyme nitrogen content was assumed to be one-sixth of the protein determined by the method of Lowry *et al.* (1951). The nitrogen content of fraction V was determined directly by the Kjeldahl procedure to permit direct comparison with the results of Hill and Smith (1958). [d] Weighted average of values for fractions I–IV (Figure 2C).

cold room and washed with starting buffer until the pH and conductivity of the influent and effluent solutions were identical.

Experiments were performed at 4° using a Sigmamotor T-8 pump and a time-indexed fraction collector. Linear gradients were produced with a simple two-chambered gradient device.

Chromatography on Columns of Sephadex G-200. Sephadex G-200 (lot 648, particle size 40–120 μ) was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Before use, it was allowed to swell for 2 weeks in two times its expected volume of 0.01 M Tris-HCl buffer (pH 8.6) (0.01 M in Tris and 0.0025 M in HCl) containing 0.001 M $MgCl_2$.

A 2.5 × 135 cm column fitted with a 1000-ml conical reservoir was filled with buffer and a slurry of Sephadex was poured into the reservoir. A hydrostatic head of 10 cm was established by adjusting the level at which the end of a flexible outlet tube was supported. A flow rate of about 15 ml/hr was obtained with the packed column, under a head of 15 cm. Effluent volume was obtained by weighing the full-fraction tubes. The plastic tubes used for collection had a constant tare within 0.05 g.

Estimations of molecular weight were performed according to the method of Auricchio and Bruni (1964), using the same column calibrated with Blue Dextran 1000 (Pharmacia, mol wt 2 × 10^6), edestin (Worthington Biochemicals, three-times recrystallized, mol wt 330,000), phycoerythrin (mol wt 290,000),[2] phycocyanin (mol wt 130,000),[2] and horse heart cytochrome *c* (Sigma, mol wt 12,500) as standards.

Preparation of Endopeptidase-Free Leucine Aminopeptidase. A CETAB supernatant solution (10 l.) was prepared from 4 kg of Yorkshire hog kidney. It was pumped at 2.4 l./hr through an 8.5 × 25 cm bed of DEAE-cellulose (Whatman DE-23) equilibrated with 0.04 M Tris–0.005 M succinic acid–0.001 M $MgCl_2$ (pH 8.6)[3] after which the column was washed with 2 l. of starting buffer. The enzyme, which was still adsorbed to the column, was eluted sharply with 2 l. of the same buffer containing 0.2 M NaCl, and the effluent was collected in 200-ml fractions. The three active fractions were located by assay and pooled (ion-exchange filtrate, Table I, step 2).

To the 660 ml of active fractions at 4° was added 207 g of $(NH_4)_2SO_4$, with constant stirring. The solution was allowed to stand for 10 min and then was centrifuged at 10,000*g* for 10 min. The supernatant solution contained 90% of the original activity, the precipitate 10%. An additional 149 g of $(NH_4)_2SO_4$ was dissolved in the supernatant solution and the latter was allowed to stand for 10 min before being centrifuged at 10,000*g* for 30 min. The second precipitate was dissolved in 100 ml of buffer (ammonium sulfate precipitate, Table I, step 3). It contained 80% of the original leucine aminopeptidase activity. This solution was divided into two 60-ml aliquots, and each was chromatographed on the 2.5 × 135 cm bed of Sephadex G-200. The active fractions were pooled (Sephadex fraction, Table I,

[2] These samples were the kind gift of Dr. H. W. Siegelman of the Beltsville Agricultural Station.

[3] The pH and conductivity of buffers were determined at room temperature.

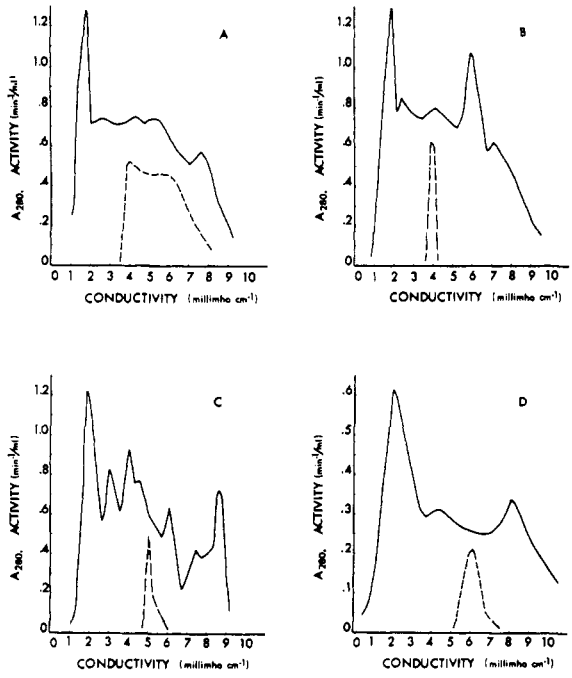

FIGURE 1: Chromatography of extracts of mongrel hog kidneys on DEAE-cellulose at pH 8.6 in a sodium chloride gradient. The distribution of protein (absorbance at 280 mμ, solid line) and enzyme activity (broken line) are shown as a function of effluent conductivity in chromatograms of extracts from pools of many kidneys (A) and from individual kidneys (B–D).

step 4) and applied directly to a 0.9 × 26 cm bed of DEAE-cellulose (Whatman DE-23) equilibrated with 0.02 M succinic acid–0.04 M Tris–0.001 M $MgCl_2$ (pH 7.4) at 66 ml/hr. The column was then washed with 80 ml of starting buffer (sufficient to wash out the pH front created by applying the sample at a higher pH and lower salt concentration than the column) and eluted with an 800-ml linear gradient to 0.05 M succinic acid–0.11 M Tris–0.001 M $MgCl_2$ (pH 7.2).

The active fractions were pooled, dialyzed against one 20-fold volume of 0.01 M ammonium acetate buffer (pH 8.6), and lyophilized to dryness. They were redissolved in 5 ml of 0.04 M Tris-HCl–0.001 M $MgCl_2$ (pH 8.6) and stored frozen in 0.1-ml aliquots.

Tests of Endopeptidase Activity. Denatured beef heart cytochrome *c* (330 mg) was prepared as described by Margoliash *et al.* (1962), and was further purified by chromatography on a 2.5 × 100 cm column of Sephadex G-50 fine beads equilibrated with 0.05 M ammonium acetate in order to remove a contaminating protein whose amino terminus was not acetylated.

The oxidized B chain of bovine insulin was prepared as described by Sanger (1949) from bovine insulin obtained from the California Biochemical Corp. Oxidized bovine pancreatic ribonuclease was prepared as described by Hirs (1956). Mercuripapain was prepared for digestion as described by Frater *et al.* (1965). Samples of rabbit actin were the kind gifts of Dr. K. Laki and Dr. Paul Adelstein of the National Institutes of Health. These proteins were denatured by boiling 4-mg/ml solutions for 10 min.

Digests contained 0.5 μmole of the protein substrate to be tested in 0.5 ml of 0.01 M Tris-HCl buffer, 0.001 M in $MgCl_2$, and 0.5–1 unit of the leucine aminopeptidase preparation to be tested. A paired control digest from which the enzyme was omitted was run simultaneously. Digestion was carried out for 18 hr at 40°.

Upon termination of digestion, 0.1-ml aliquots of the digests were evaporated to dryness and redissolved in 10 μl of water. They were then spotted on a thin layer of cellulose (Eastman Chromagram 6064) and chromatographed in 1-butanol–acetic acid–water (60:12:15) (Von Arx and Neher, 1963). The solvent front was allowed to rise 15 cm beyond the origin. The sheet was then dried for 15 min at 60° and dipped in the ninhydrin reagent described above. Development was carried out for 20 min at 60°. When 0.01 μmole of arginine, the amino acid with the lowest R_F in this system, was added to a 0.1-ml aliquot of a blank digest, it was easily detected as a spot ahead of the denatured protein which remained at the origin.

The remainder of each digest (0.4 ml) was chromatographed on a 0.9 × 25 cm bed of Sephadex G-50 fine beads equilibrated with 0.002 M collidine acetate buffer (pH 8.0). The fractions were examined for A_{220} and for reaction with ninhydrin. In a test of the sensitivity of the system, 0.01 μmole of leucine added to a control digest was easily detected by ninhydrin.

Results

Chromatographic Behavior of Enzyme from Mongrel and Pure-Strain Hog Kidneys. The four chromatograms in Figure 1 illustrate the results of preliminary studies which were designed to establish the conditions required for successful gradient chromatography of material derived from detergent precipitation of homogenates of kidney. In Figure 1A an aliquot of CETAB supernatant solution derived from a homogenate of 20 mongrel hog kidneys was chromatographed on a 1.8 × 25 cm column of DEAE-cellulose (Whatman DE-50 Floc) equilibrated with 0.04 M Tris–0.005 M succinic acid–0.001 M $MgCl_2$ (pH 8.6). Elution was accomplished with a 400-ml linear gradient to 0.2 M NaCl in the same buffer. A flow rate of 60 ml/hr was employed and 4-ml fractions were collected. In Figure 2B–D, aliquots of CETAB supernatant solution derived from individual kidneys from the same batch of mongrel hog kidneys were examined in an identical manner.

The broadness of the activity peak obtained with mixed mongrel hog kidneys (Figure 1A) suggested that the enzyme was not homogeneous. Individual kidneys from the same batch, on the other hand, yielded sharp peaks of activity, but these emerged at salt concentrations varying in conductivity from 3.9 in example B to 5.9 mmho cm^{-1} in example D (see Figure 1). In fact, five significantly different positions were observed in the six kidneys thus examined. Fractions from examples B and D were rechromatographed, and each emerged in its original position.

Enzyme activity from renal extracts of each of the three inbred strains examined (Yorkshire, Hampshire, and Spotted Poland China) emerged in a sharp peak at

a conductivity of 4.3 mmho cm^{-1} in this chromatographic system. This identity of chromatographic position probably reflects the known close genetic relationship of these strains. Although all of them provided chromatographically suitable starting material, the enzyme from Yorkshire kidneys appeared to be slightly more stable. For this reason, kidneys from this strain were used for all further studies.

Properties of Purified Enzyme. The results of the purification scheme developed are summarized in Table I and the elution diagrams obtained in the chromatographic steps are illustrated in Figure 2. An over-all purification of 1200-fold was achieved (using the CETAB supernatant solution as a base line), with a yield of 50% in five steps. The final specific activity (C') ranged from 98 to 158 min^{-1}/mg of protein N in five preparations.

Although the enzyme activity emerged as a single component when chromatographed on DEAE-cellulose at pH 8.6 (Figure 1B–D) and on Sephadex G-200 at pH 8.6 (Figure 2B) or pH 7.5 (*vide infra*), chromatography on DEAE-cellulose in a shallow gradient at pH 7.5 (Figure 2C) gave evidence of multiple forms of the enzyme. The partially resolved active components were designated I–IV, as indicated in Figure 2C, and were separately examined for substrate specificity and endopeptidase activity as described below.

Endopeptidase Activity. When enzyme fractions from the early stages of purification (through the ammonium sulfate precipitation) were used, both the column and thin-layer chromatographic tests easily detected release of amino acids from protein substrates with amino-terminal sequences refractory to leucine aminopeptidase. This is illustrated in Figure 3A,B, where the elution patterns from Sephadex G-50 chromatography of undigested denatured cytochrome *c* (A) and of denatured cytochrome *c* digested with step 2 leucine aminopeptidase (B) may be compared. Undigested denatured cytochrome *c* emerged from the column in a single, colored peak. Only a small amount (0.01 leucine equiv/mole of cytochrome *c* applied) of ninhydrin-positive material was present in the position in which amino acids would emerge from the column. Digested cytochrome *c*, however, emerged in three peaks (as detected by 220-mμ absorbance) followed by a large peak (10 leucine equiv/mole of cytochrome *c* applied) of ninhydrin-positive material in the amino acid position. In contrast, when enzyme-containing fractions from later purification steps were employed (Figure 3C) the chromatograms of the digests were identical with those obtained with undigested cytochrome *c*. Since the acetylated amino terminus of cytochrome *c* renders it refractory to leucine aminopeptidase digestion, any hydrolysis must be attributed to endopeptidase contamination. Thus, the data show that crude fractions from the present method of preparation contain endopeptidase contamination, whereas such contamination was not detectable in later stages. Some hydrolysis of cytochrome *c* was observed by Frater *et al.* (1965), using a less sensitive detection system, even with their purest preparations.

The extension of these observations to other proteins

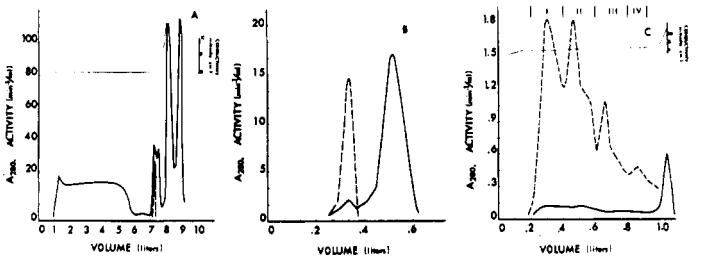

FIGURE 2: Chromatographic steps in the isolation of endopeptidase-free leucine aminopeptidase. The distribution of protein (A_{280}, thick solid line), activity (broken line), and conductivity (fine solid line) in the chromatographic steps of leucine aminopeptidase purification are shown. (A) Step 1, Table I, ion-exchange filtration on DEAE-cellulose at pH 8.6. (B) Step 4, Table I, chromatography on Sephadex G-200. (C) Step 5, Table I, gradient chromatography on DEAE-cellulose at pH 7.5. The Roman numerals above the elution profile indicate the manner in which fractions were pooled. For details, see Methods.

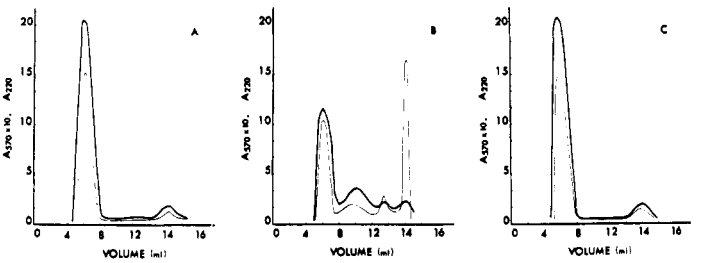

FIGURE 3: Chromatography of digests of denatured beef heart cytochrome *c* on Sephadex G-50. The distributions of A_{220} and ninhydrin-positive material (A_{570}) are shown as a function of effluent volume. Marker amino acids (except for the aromatic ones) emerged from the column at 14 ml. (A) Cytochrome *c* incubated for 18 hr without addition of enzyme. (B) Cytochrome *c* incubated for 18 hr with the enzyme-containing fractions from step III, Table I. (C) Cytochrome *c* incubated for 18 hr with step 5 leucine aminopeptidase, fraction I (Figure 2C).

whose N-terminal sequences render them immune (Frater *et al.*, 1965) to leucine aminopeptidase digestion (rabbit actin, samples 1 and 2 (acetylated amino terminus), and mercuripapain (N-terminal sequence = Ile-Pro-)) as well as to two presumably susceptible substrates (the oxidized B chain of bovine insulin and oxidized bovine pancreatic ribonuclease) is summarized in Table II which contrasts the amount of amino acid released from each of these substrates by a crude fraction of leucine aminopeptidase and by the final product of purification. The crude fraction hydrolyzed every one of the tested substrates that was immune to leucine aminopeptidase. Thus, endopeptidases to which these substrates are susceptible are present in kidney. The final product of purification, on the other hand, was unable to hydrolyze any of these proteins but rapidly released amino acids from the oxidized B chain of bovine insulin. Examination of the amino acids released as a function of time, using thin-layer chromatography, showed that the order of release corresponded to the order of the first five amino acids in the amino-terminal sequence of the protein. Thus, although an active exopeptidase, the purified enzyme does not contain endo-

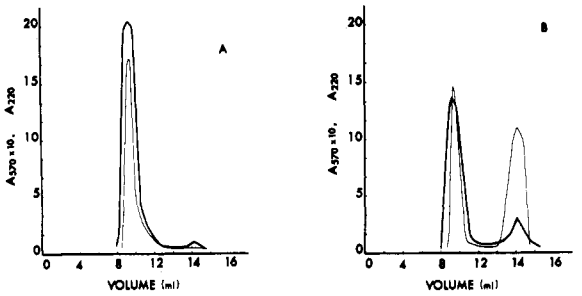

FIGURE 4: Chromatography of digests of the oxidized B chain of bovine insulin on Sephadex G-50. The distribution of A_{220} and ninhydrin-positive material (A_{570}) are shown as a function of effluent volume. Chromatographic conditions as in Figure 3. (A) Insulin B chain incubated for 12 hr without addition of enzyme. (B) Insulin B chain digested for 12 hr with step 5 leucine aminopeptidase fraction I (Figure 2C).

peptidases which can detectably hydrolyze three protein substrates whose sequences contain a representative array of peptide bonds.

The nature of the digestion of oxidized insulin B chain by leucine aminopeptidase was further investigated by chromatographing the products of digestion on Sephadex G-50 in a manner exactly analogous to that described with denatured cytochrome *c*. One such chromatogram (after 12-hr digestion) is illustrated in Figure 4B. Although a total of 5 moles of leucine equiv/mole of applied insulin chain above the control level had been released, no perceptible change in the position of the main A_{220} component was observed, and no slower moving A_{220} components were produced. The amino acids present in the ninhydrin peak were phenylalanine, valine, asparagine, glutamine, histidine, and leucine. A trace of cysteic acid was also detectable. These amino acids are the first seven in the known sequence of this protein (Sanger, 1952). Similar studies were made after 1-, 2-, 6-, and 12-hr hydrolyses. At no time were peptide fragments detected.

Oxidized bovine pancreatic ribonuclease was not hydrolyzed by this purified enzyme (Table II, 6), even though hydrolysis by other leucine aminopeptidase preparations has been reported (Frater *et al.*, 1965). The reason for this is not clear at present, but may be related either to the known low activity of leucine aminopeptidase toward peptides containing lysine as the amino-terminal residue (Smith *et al.*, 1952) or to an influence of substrate configuration on the susceptibility to hydrolysis of proteins as opposed to peptides.

In Table III are listed the specific activities of the four fraction pools from the final DEAE-cellulose chromatography toward five substrates. All four hydrolyze leucinamide at a rapid rate, although the later fractions (pools III and IV) have significantly lower specific activities than the earlier fractions with respect to this substrate, perhaps reflecting a lower degree of purity. All of the fraction pools also hydrolyze the phenylalanine chain of insulin at approximately the same rate relative to their leucinamide hydrolyzing activity. None of them hydrolyze either D-leucylglycine or N-acetylleucylglycine at a perceptible rate. In all of these respects they resemble earlier leucine aminopeptidase preparations.

TABLE II: Digestion of Protein Substrates by Leucine Aminopeptidase Containing Fractions.

Substrate	Amino Acids Released by Crude Leucine Aminopeptidase[a] (μmoles of leucine equiv/μmole of substrate per 12 hr)[c]	Amino Acids Released by Purified Leucine Aminopeptidase[b] (μmoles of leucine equiv/μmole of substrate per 12 hr)[c]
Bovine heart cytochrome *c*	10.4	−0.01[d]
Mercuripapain	6.7	−0.002[d]
Rabbit actin, sample 1[e]	33.0	0.02
Rabbit actin, sample 2[f]	35.6	−0.005[d]
Oxidized B chain of bovine insulin	28.0	10.7
Oxidized bovine pancreatic ribonuclease	14.0	0.01

[a] From step 3 of Table I. [b] From step 5 of Table I (fraction I of Figure 2C). [c] This value is derived from the quantitative ninhydrin analysis of the fractions from Sephadex G-50 chromatography of digests of the substrate listed, as described in detail under Methods. The values given are the total leucine equivalents of ninhydrin-positive material found in the amino acid position of the digest chromatograms minus the total leucine equivalents of ninhydrin-positive material in the amino acid position of the corresponding control. [d] These slightly negative values are not analytically significant. [e] This sample was the kind gift of Dr. K. Laki, National Institutes of Health. [f] This sample was the kind gift of Dr. P. Adelstein, National Institutes of Health.

Distinct differences are present, however, in the relative rates at which the different fractions hydrolyzed leucyl-*p*-nitroanilide and leucinamide (Table III, column 3), the ratio of the values obtained being 4.3 and 4.5 for fractions I and II and 2.0 and 2.2 for fractions III and IV.

The elution position (from Sephadex G-200) of leucine aminopeptidase component I is compared with the elution position of various proteins of known molecular weight in Table IV. Identical results were obtained whether the chromatography was carried out at pH 8.6 or 7.5, and the positions of the other leucine aminopeptidase components were identical with that of leucine aminopeptidase I. When these data are treated according to the method of Auricchio and Bruno (1964),

TABLE III: Specific Activity of Highly Purified Leucine Aminopeptidases[a] toward Various Substrates.

Fraction Pool[b]	Leucinamide (min^{-1}/mg per min)[c]	Leucine-*p*-nitroanilide (μmoles/mg per min)[d]	Oxidized B Chain of Insulin (μmoles of amino acid/mg per min)[e]	D-Leucylglycine (μmole/mg per min)[f]	*N*-Acetylleucylglycine (μmole/mg per min)[f]
I	127	550	0.45	0	0
II	117	530	0.51	0	0
III	99	200	0.40	0	0
IV	80	180	0.28	0	0

[a] The enzyme preparations used were obtained from step 5 of Table I. Each sample was activated with Mn^{2+} as described under Methods before assay. All assays were run in 0.1 M Tris-HCl buffer containing 0.001 M $MnCl_2$, at 40°, and specific activities were calculated on the basis of Kjehldahl nitrogen determinations. [b] As indicated in Figure 2C. [c] Substrate concentration 0.05 M (pH 8.6). [d] Substrate concentration 1.66×10^{-3} M (pH 7.8). A molar extinction of 9260 for *p*-nitroaniline was assumed (Wachsmuth *et al.*, 1966). [e] Described in detail under methods. [f] Substrate concentration 0.01 M (pH 8.6).

an estimate of 300,000 is obtained for the molecular weight of the enzyme.

Discussion

The method described in this paper can reproducibly provide leucine aminopeptidase of high specific activity; about 30 mg of purified enzyme having a proteolytic coefficient of 98–158 min^{-1}/mg of N is obtained from 4 kg of hog kidneys, and the product is stable for at least 1 year in the frozen state. This specific activity appears to be slightly higher than those reported for pooled preparations obtained by other methods (90–100), although peak fractions with specific activities of 150 min^{-1} were obtained after chromatography on DEAE-cellulose (Frater *et al.*, 1965; Folk *et al.*, 1959).

Improvement over older methods depended upon two major factors. First, ion-exchange chromatography and gel filtration were not available at the time of the last extensive investigation of the enzyme (Smith *et al.*, 1952). Application of these high-resolution procedures, however, required the recognition of heterogeneity of leucine aminopeptidase from different sources of hog kidney. Although the nature of this intersource heterogeneity has not been established, its practical consequence is to cause the elution of leucine aminopeptidase from DEAE-cellulose with a wide spectrum of other proteins, thus impairing effective chromatographic purification when mixed kidneys are used as a source of enzyme. This problem was overcome by the use of kidneys from a commercially available inbred strain of hogs. Although the potential hazards of mixing sources has long been recognized, this is, to our knowledge, the first demonstration that the pooling of individual specimens can produce heterogeneity sufficient to hinder enzyme purification seriously and it seems likely that such difficulties will be encountered with increasing frequency as procedures of high resolution are applied more often to the early stages of enzyme purification from slaughterhouse materials.

The second advantage of the present method over its predecessors lies in the use of an initial extract of kidney that is both easier to prepare and easier to purify than the acetone powders previously employed. Since renal cathepsins occur in a particulate fraction, and since the pellet from the first centrifugation in the present purification procedure contained plentiful endopeptidase activity, it is likely that the successful removal of endo-

TABLE IV: Elution Positions of Proteins of Known Molecular Weight and of Leucine Aminopeptidase from a Standard Column of Sephadex G-200.[a]

Substance	Mol Wt	Elution Vol (ml)
Blue Dextran 1000	2,000,000	274
Edestin	330,000[b]	360
Phycoerythrin	290,000[c]	380
Phycocyanin	130,000[c]	484
Equine cardiac cytochrome *c*	12,500[d]	618
Step 5 leucine aminopeptidase fraction I (at pH 8.6)		368
Step 5 leucine aminopeptidase fraction I (at pH 7.5)		366

[a] For a detailed description of the conditions of chromatography, see Methods. The elution position given is that of the peak of the component in question, located, where possible, by its characteristic spectral absorption or activity, and where not, by the position of maximum absorption at 280 mμ. The positions of the standard proteins were the same at pH 7.5 as at pH 8.6. [b] Svedberg (1937a,b). [c] Svedberg and Eriksson-Quensel (1936). [d] Svedberg (1937a,b).

peptidases by the present method depends in part on the early removal of the particulate fractions of the renal cortical cell by detergent precipitation. An additional advantage of the replacement of acetone powders by supernatant fraction is the preservation of leucine aminopeptidase M (Pfleiderer and Celliers, 1963) activity in the precipitate from detergent precipitation. This enzyme is apparently completely destroyed during the preparation of acetone powders.

The usefulness of the leucine aminopeptidase prepared by the method described here is suggested not only by its high specific activity but also by the evidence which indicates that endopeptidase activity has been reduced to an insignificant level, or, indeed, eliminated entirely. In principle, of course, it is impossible to prove the absence of endopeptidase activity rigorously, and every application of the enzyme which depends for success on the absence of internal splits will require specific tests on the particular substrate being used. Nevertheless, the absence of detectable hydrolysis of four proteins whose amino-terminal sequences render them immune to aminopeptidase digestion, but which contain a wide variety of peptide bonds presumably susceptible to endopeptidase digestion (Table II), strongly suggests that endopeptidase contamination has been reduced to an insignificant level (less than 0.02 μmole of total amino acid released/μmole of substrate). The evidence also documents that the digestion of the susceptible substrate, oxidized insulin B chain, by the present preparation proceeds from the amino terminus only. Similar data obtained in another laboratory (J. Potts and L. Deftos, personal communication) show that parathyroid hormone is degraded from the amino terminus by this enzyme and that the sequence obtained by quantitative amino acid analysis of timed hydrolysates agrees with the known sequence deduced by other means. As with insulin, no detectable change in the size of the hormone was observed after several amino acids had been removed.

The chromatographic heterogeneity of leucine aminopeptidase derived from kidneys of inbred hogs (Figure 2C) is to be distinguished from that observed when non-inbred hog kidneys are employed (Figure 1A). Extracts of mixtures of mongrel hog kidneys yield very broad peaks when chromatographed at pH 8.6, whereas, under these conditions, extracts of inbred hog kidneys yield sharp peaks, heterogeneity only becoming apparent when chromatography is performed at pH 7.5. The significance of the multiplicity of leucine aminopeptidase components observed in the case of inbred hog kidneys is not known, but the validity of the chromatographic heterogeneity is indicated by differences in the substrate specificity of the separated components (Table III). Taken together, the existence of multiple components and the very large molecular weight of the enzyme suggest that it may have a subunit structure. Simple aggregation is an unlikely explanation of these properties, because the molecular size of the protein, as judged by Sephadex chromatography, is the same at pH 8.6, where it is homogeneous by ion-exchange chromatography, and pH 7.5, where heterogeneity by ion-exchange chromatography is evident.

Analyses of these components by atomic absorption spectroscopy have consistently demonstrated the presence of stoichiometrically significant quantities of zinc and the absence of significant amounts of manganese. This is of particular interest in light of the beneficial effect of zinc on the crystallization of the very similar leucine aminopeptidase of beef lens observed by Hanson *et al.* (1965). The possible role of zinc as an active constituent of leucine aminopeptidase and the nature of the heterogeneity of the enzyme isolated from inbred kidneys are the subjects of current investigation.

Acknowledgments

The authors thank Mr. Hugh Foster, Mrs. Janet Carlson, and Mrs. Marion Nau for skilled technical assistance, and Dr. Herbert A. Sober for his advice and support.

References

Auricchio, F., and Bruni, C. B. (1964), *Biochem. Z. 340*, 321.

Fasold, H., Linhart, P., and Turba, F. (1962), *Biochem. Z. 336*, 182.

Folk, J. E., Gladner, J. A., and Viswanatha, T. (1959), *Biochim. Biophys. Acta 36*, 356.

Frater, R., Light, A., and Smith, E. L. (1965), *J. Biol. Chem. 240*, 253.

Hanson, H., Glaeser, D., and Kirschke, H. (1965), *Z. Physiol. Chem. 340*, 107.

Hill, R. L., and Smith, E. L. (1958), *J. Biol. Chem. 231*, 117.

Hill, R. L., Spackman, D. H., Brown, D. M., and Smith, E. L. (1958), *Biochem. Prepn. 6*, 35.

Himmelhoch, S. R., and Peterson, E. A. (1966), *Anal. Biochem. 17*, 383.

Himmelhoch, S. R., Sober, H. A., Vallee, B. L., Peterson, E. A., and Fuwa, K. (1966), *Biochemistry 5*, 2523.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

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

Margoliash, E., Kimmel, J. R., Hill, R. L., and Schmitt, W. R. (1962), *J. Biol. Chem. 237*, 2148.

Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 1.

Pfleiderer, G., and Celliers, P. G. (1963), *Biochem. Z. 339*, 186.

Sanger, F. (1949), *Biochem. J. 43*, 126.

Sanger, F. (1952), *Advan. Protein Chem. 7*, 2.

Smith, E. L., and Hill, R. L. (1960), *Enzymes 4*, 37.

Smith, E. L., and Spackman, D. H. (1955), *J. Biol. Chem. 212*, 271.

Smith, E. L., Spackman, D. H., and Polglase, W. J. (1952), *J. Biol. Chem. 199*, 801.

Spackman, D. H., Brown, A., and Smith, E. L. (1955), *J. Biol. Chem. 212*, 255.

Svedberg, T. (1937a), *Nature 139*, 1051.

Svedberg, T. (1937b), *Chem. Rev. 20*, 81.

Svedberg, T., and Eriksson-Quensel, I.-B. (1936), *Tab. Biol. 11*, 351.

Von Arx, E., and Neher, R. (1963), *J. Chromatog. 12*, 329.

Wachsmuth, E. D., Fritze, I., and Pfleiderer, G. (1966), *Biochemistry 5*, 175.

Dinitrophenylation of Glycogen Phosphorylase. I. Preparation and Properties of Active Dinitrophenyl Derivatives*

George Philip and Donald J. Graves†

ABSTRACT: Dinitrophenylation of phosphorylase *b* with an eightfold molar excess of 2,4-dinitrofluorobenzene (DNFB) results in inactivation of the enzyme, and subsequent analyses of the protein showed modification of four to five groups. ε-Amino groups of lysine and SH groups of cysteine were modified. In the presence of either α-D-glucose 1-phosphate (glucose-1-P) or adenosine 5′-monophosphate (AMP), inactivation is retarded and in the presence of both, 75% of the activity could be retained with modification of 3–3.5 groups. When glucose-1-P or AMP was present during dinitrophenylation, their respective binding sites were preserved as indicated by kinetic studies DNP-phosphorylase *b*, prepared in the presence of glucose-1-P or AMP or both, could be converted into crystalline DNP-phosphorylase *a* derivatives. DNP-phosphorylase *a* prepared from phosphorylase *b* dinitrophenylated in the presence of glucose-1-P and AMP was found to be electrophoretically homogeneous and sedimented essentially as a single component in the ultracentrifuge. No change in K_M for glucose-1-P and AMP was observed with this derivative in comparison with the unmodified enzyme but a clear difference existed in the K_M for glycogen.

In order to delineate the importance of specific amino acid residues in phosphorylase structure and activity, the susceptibility of this enzyme to a chemical modifying agent, DNFB,[1] has been tested. Although reactions of phosphorylase with chemical agents have not been used extensively, Madsen and Cori (1956) showed that modification of nine cysteinyl residues of phosphorylase *b* dimer resulted in formation of an inactive monomer. More recently, studies of phosphorylase with other sulfhydryl reagents (Kudo and Shukuya, 1964; Damjanovich and Kleppe, 1966; Damjanovich *et al.*, 1967) further defined the role of cysteinyl residues in catalysis. The involvement of lysyl residues in structure and activity has been demonstrated by Huang and Madsen (1966); their studies with cyanate showed that carbamylation of 23 ε-amino groups of lysine in phosphorylase *b* resulted in enzyme inactivation and dissociation.

Dinitrophenylation of phosphorylase was chosen since ribonuclease (Hirs, 1962), aldolase and transaldolase (Rowley *et al.*, 1964; Kowal *et al.*, 1965), and fructose 1,6,-diphosphatase (Pontremoli *et al.*, 1965), all enzymes like phosphorylase in that their substrates are anionic, could either be inactivated or converted into a different catalytic form by dinitrophenylation of a small number of amino acid residues.

The work reported herein, shows that the lysyl residues can be preferentially modified under specific conditions and that inactivation can be accomplished by modification of a small number of groups. Under somewhat different experimental conditions, inactivation results largely from modification of sulfhydryl groups (Gold, 1968).

Experimental Section

Materials. Phosphorylase *b* from rabbit skeletal muscle was prepared according to the procedure of Fischer and Krebs (1958) but mercaptoethanol was substituted wherever cysteine was required. Three-times-recrystallized phosphorylase *b* was used in this work. Phosphorylase *b* was converted into phosphorylase *a* by using phosphorylase *b* kinase as described by Fischer and Krebs (1962). Enzyme used for dinitrophenylation was first dialyzed for 2 days against 0.025 M Tris-HCl buffer (pH 7.6) at 3–4° to remove mercaptoethanol. The specific activities of a typical preparation of this enzyme were 1700 and 1250 Cori units/mg, respectively, when measured according to the procedure of Illingworth and Cori (1953) in 0.015 M cysteine–0.02 M glycerophosphate (pH 6.8) and in 0.0125 M

* From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010. *Received September 25, 1967.* Journal Paper J-5789 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 1510. This research was supported by a research grant (GM-09587) from the National Institutes of Health, U. S. Public Health Service.

† Recipient of a research career development award of the U. S. Public Health Service (Grant GM6753).

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DNFB, 2,4-dinitrofluorobenzene; PMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).