Purification and N-terminal determination of crystalline pepsin

The situation with regard to the chemical and enzymic homogeneity of pepsin is not clear, as witnessed by several reports[1,2,3], and we have therefore found it worth-while to reinvestigate this question. A sensitive criterion of chemical homogeneity is offered by N-terminal determination, but in the case of pepsin a special difficulty is encountered owing to the fact that small peptides, presumably formed by autolysis of the enzyme, contaminate the preparation and thus obscure the result of the N-terminal determination. However, it was found that by passing a pepsin solution through a column of Dowex-50 in the acid form, the larger part of the contaminating peptides were retained by the resin whereas the enzyme passed through. The preparation thus obtained has been used for N-terminal determinations.

100 g Dowex-50 (15–30 mesh) were converted into the acid form[4], brought on a column (diam. 2.0 cm) and rinsed with dilute HCl (pH 2.8) until the effluent was free of material absorbing in the ultra-violet, and the pH was 2.8. 500 mg crystalline pig pepsin[5] (specific activity, 0.13[6]) were dissolved in 100 ml distilled water and a small amount of insoluble material centrifuged off. The clear supernatant was acidified to pH 2.8 (glass electrode). A slight turbidity disappeared on the addition of 50 ml dilute HCl. The pepsin solution was passed through the column at a rate of approx. 7 ml/min and the column subsequently rinsed with 70 ml dilute HCl (pH 2.8). In order to minimize autolysis, the effluents were run directly into 20 ml acetic acid-sodium acetate buffer (total molarity 0.16), pH 5.3. The effluents were quickly frozen and lyophilized. The recovery of nitrogen was 94%, and of pepsin activity, 105%. The specific activity was 0.17. A part of the freeze-dried product, corresponding to 40 mg pepsin, was dissolved in 10 ml dilute HCl (pH 2.8) and passed a second time through a Dowex-50 column (17 g Dowex-50, acid form; diam., 0.9 cm) at a rate of approx. 2 ml/min. The column was rinsed with 10 ml dilute acid. The effluent was immediately neutralized and lyophilized. All operations were carried out in the cold room and as quickly as possible.

The N-terminal determination was carried out by the phenyl thiocarbamyl (PTC) method as previously described[7]. A sample of the lyophilized pepsin prepared as above corresponding to 12.4 mg protein was dissolved in pyridine–water (1:1), the solution made alkaline with $NaHCO_3$ and 20 μl phenyl *iso*thiocyanate added. The solution was left at room temperature for 2 h and then extracted 3 times with equal volumes of ethyl acetate. The PTC-protein was precipitated from the aqueous solution with 9 vol. acetone and dried. To the dry product was added 1 ml *N* HCl. The suspension was kept at 100° C for 1 h and afterwards extracted 3 times with 1 ml ethyl acetate. The extracts were combined, the solvent evaporated, and the dry residue dissolved in 0.1 ml ethylene chloride.

Aliquots of the ethylene chloride solution were taken for paper chromatographic identification of the phenyl thiohydantoins (PTH) along with samples of authentic PTH's. The solvent system used was formamide/xylene[8] and the spots were visualized both with the iodine azide-starch reaction[9] and on the fluorescent screen[8]. By far the strongest spot had an R_F value corresponding to PTH-leucine or PTH-isoleucine. In addition there were weaker spots which have not been identified with certainty. Parallel runs with unknown, PTH-leucine and PTH-isoleucine and mixed spots showed clearly the identity of the N-terminal of pig pepsin with PTH-isoleucine.

For quantitative determination[8] of the N-terminal the spot was eluted from the paper and the U.V.-absorption of the eluate measured at 268 mμ where the PTH's have maximum absorption. Correcting for losses during ring closure to PTH and extraction (approx. 20%) and during paper chromatography (approx. 20%), 1 mole of PTH-isoleucine was found to correspond to 40,000 g of pepsin.

A sample of the Dowex-treated material corresponding to 39 mg pepsin was dissolved in 1 ml formic acid, 0.02 ml H_2O_2 (30% w/w) added and the mixture left for 1 h at room temperature[10]. The performic acid was then destroyed by the addition of 1 ml water, and the oxidized pepsin precipitated with 9 vol. acetone. The precipitate was washed with acetone and dried, and the dry product solubilized in aqueous 6 *M* urea and treated for N-terminals as described above. Since the expected PTH-cysteic acid[11] is too strongly polar to allow identification in the regular procedure, paper electrophoresis was resorted to for the identification. The acid solution used in the splitting off of the N-terminals was brought to pH 4 with K_2CO_3 and a sample of the solution subjected to paper electrophoresis in an acetic acid-potassium acetate buffer (total molarity 0.04), pH 4, along with authentic samples of PTH-cysteic acid. No PTH-cysteic acid was detectable in the test sample.

In conclusion, our results show that isoleucine is the sole N-terminal amino acid of swine pepsin. The value 1 mole/40,000 g pepsin preparation, fits in reasonably well with the accepted molecular weight for pepsin of 35,500. Our findings support those of VAN VUNAKIS AND HERRIOTT[12], who identified isoleucine as the N-terminal, and disagree with those of WILLIAMSON AND PASSMAN[13], who found leucine. Although the difference in R_F value between PTH-leucine and PTH-isoleucine is small, the mixed spot technique has allowed us to identify the N-terminal unambiguously as isoleucine.

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Reversible reduction of thioctamide catalyzed by the α-ketoglutaric dehydrogenase complex

The pyruvic and α-ketoglutaric (KG) dehydrogenases of mammalian tissues are complex units of high molecular weight ($4 \cdot 10^6$ and $2 \cdot 10^6$ g/mole respectively)[1,2]. Under the electron microscope*, the latter appears as an essentially spherical particle of 120 A diameter which is consistent with the molecular weight calculated from sedimentation and diffusion constants. Both of these enzyme complexes contain tightly-bound, non-dialyzable 6,8-thioctic acid (α-lipoic acid) or a derivative of it active in the microbiological assay[2,3]. Direct evidence that thioctic acid functions in the oxidation of α-ketoacids has been obtained from studies on bacterial preparations[4,5]. One of the intermediate steps postulated in the sequence of reactions is the oxidation of dithioloctanoate by DPN (Reaction 1)

$$T(SH)_2 + DPN^+ \rightleftarrows TS_2 + DPNH + H^+ \qquad (1)$$

The presence of this enzymic activity in purified fractions from *E. coli* has been demonstrated by coupling it to the reduction of pyruvate with lactic dehydrogenase and measuring the decrease in $-SH$[6].

We have now found that the KG dehydrogenase purified from hog hearts[2] catalyzes the reversible oxidation of reduced DPN (DPNH) by 6,8-thioctate and by 6,8-thioctamide**. The reaction can be demonstrated readily in both directions by measuring the absorption of DPNH at 340 mμ (Fig. 1). (+) Thioctate is active while (—) thioctate is inactive in the reaction. The presence of the inactive isomer had no effect on the velocity of the reaction with the (+) isomer.

There are significant differences in the activity with thioctate and thioctamide. The pH optimum for the reaction with thioctamide is 7.1, which is about the same as for KG oxidation by DPN[7]. With thioctate as substrate, the activity increases sharply on decreasing the pH from 7.0 to 6.0. The concentration for half-maximal velocity with DL-thioctamide is $6 \cdot 10^{-4} M$, while maximal rates are not obtained with DL-thioctate even at $6 \cdot 10^{-3} M$. The rates of KG oxidation and of DPNH oxidation by thioctate or thioctamide are shown in Table I. It is seen that the partial reactions proceed at a faster rate than the over-all KG oxidation. The fact that, compared with the reduction of thioctic acid, the reaction with thioctamide has a lower K_m and a pH optimum closer to that of KG dehydrogenase tentatively suggests that the natural bound cofactor

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