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Preparation of water-insoluble, enzymatically active derivatives of subtilisin type Novo by cross-linking with glutaraldehyde

A variety of methods has been described for the coupling of proteins to insoluble carriers without loss of biological activity. In some cases the protein itself was made insoluble through a cross-linking reaction by means of a chemical reagent[1]. Glutaraldehyde was used by QUIOCHO AND RICHARDS[2] and by HABEEB[3] to prepare water-insoluble, enzymatically active derivatives of carboxypeptidase and trypsin, respectively. In the present report glutaraldehyde has been used to prepare an insoluble derivative of subtilisin type Novo (EC 3.4.4.16), which retained 10–15% of the specific activity of the untreated subtilisin.

Crystalline subtilisin type Novo, batch ch 31c, was a gift from the Novo Pharmaceutical Company. 50-mg aliquots of this enzyme were dissolved in 1.0 ml of 0.1 M

TABLE I

THE FORMATION OF INSOLUBLE SUBTILISIN TYPE NOVO, BY REACTION WITH GLUTARALDEHYDE UNDER VARIOUS CONDITIONS

Subtilisin type Novo (50 mg) was dissolved in 1 ml acetate buffer (pH 5.0) or phosphate buffer (pH 6.0 to 8.0) and ammonium sulphate or acetone was added as shown. The reaction was initiated by addition of 80 μl of 25% glutaraldehyde at room temperature.

Reaction mixture										
Acetate or phosphate buffer, pH	5.0	5.0	5.0	5.0	6.0	7.0	8.0	5.0	5.0	5.0
Ammonium sulphate, percentage satd.	0	30	40	50	50	50	50	0	0	0
Acetone, ml/ml	0	0	0	0	0	0	0	0.6	0.8	1.0
Time of reaction, min	30	30	30	30	30	30	30	60	60	60
Reaction product										
Percentage yield by weight	0	12.6	32.8	48.6	21.6	63.6	73.2	42.0	48.0	42.0
Percentage yield of enzyme activity	0	1.6	4.7	6.1	2.9	4.6	1.7	4.8	4.1	5.0
Specific activity in percentage of specific activity of native enzyme	0	13.4	14.4	12.5	13.4	7.2	2.3	11.4	8.0	11.8

acetate or phosphate buffer of the desired pH and ammonium sulphate or acetone was added as indicated in Table I. After addition of 0.08 ml of a 25% solution of glutaraldehyde (commercial product from Fluka, A.G.) the reaction mixture was stirred for 30 min or 1 h at room temperature. The precipitate was isolated by centrifugation and

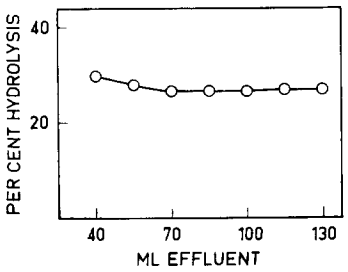

Fig. 1. Continuous hydrolysis of casein by insoluble subtilisin type Novo. On top of a column of cellulose powder (0.6 × 6 cm, 40°) a suspension of 10 mg insoluble subtilisin type Novo, was placed. A 0.5% casein solution (pH 7.8) was then passed through the column with a flow rate of approx. 9 ml/h. Fractions of 15 ml were collected and the extent of hydrolysis estimated by the biuret test.

washed with 0.15 M NaCl solution until no more colour could be removed, with 0.1 M phosphate buffer (pH 8.0), until practically free of soluble enzymatic activity, and with water until free of salt. The brownish coloured enzyme derivative was used in a water suspension and was stored at 4°. The concentration of the derivative was determined from the content of solid matter in the suspension and the enzymatic activity was determined with the pH-stat technique[4] at pH 8 and 30°, using casein as substrate.

As shown in Table I, glutaraldehyde precipitated the subtilisin in the presence of ammonium sulphate or acetone. The best yields of active enzyme derivative were obtained at pH 5 and 50% ammonium sulphate, but the yields obtained by addition of acetone were only slightly lower. The insoluble enzyme derivative was stable for

TABLE II

AMINO ACID ANALYSIS OF INSOLUBLE SUBTILISIN TYPE NOVO, AFTER REACTION WITH GLUTARALDEHYDE

The hydrolysis was performed with 6 M HCl in a sealed evacuated tube for 24 h at 110° and the amino acid analysis was made on a Beckmann-Spinco amino acid analyzer, Model 120, modified to complete a run in 3.5 h. For comparison the amino acid composition of the unmodified enzyme is included.

Amino acid	*Insoluble enzyme*	*Native enzyme*
Lysine	6.9	11
Histidine	5.5	6
Arginine	1.7	2
Aspartic acid	27.7	28
Threonine	12.2	13
Serine	31.4	37
Glutamic acid	14.8	15
Proline	14.5	14
Glycine	33.6	33
Alanine	38.0	37
Valine	27.0	30
Methionine	4.6	5
Isoleucine	10.2	13
Leucine	13.8	15
Tyrosine	9.3	10
Phenylalanine	3.2	3

several weeks, and a mixture of insoluble enzyme with cellulose powder could be used to digest proteins in a chromatographic column. Fig. 1 shows how such a column gave a constant degradation of a casein solution, which was slowly flowing through the column for a period of 10 h. The insoluble enzyme derivatives were observed to degrade casein, clupein, gelatine, *N*-benzoyltyrosine ethyl ester and toluene sulphonyl-L-arginine methyl ester with relative rates of splitting similar to the relative rates obtained with the unmodified subtilisin.

Amino acid analysis, performed after 24 h of hydrolysis with 6 M HCl at 110°, indicated the content of all amino acid residues to be unchanged except that of lysine (Table II). The content of this amino acid was decreased from 11 residues per mole in unmodified enzyme to 7 residues per mole after reaction with glutaraldehyde, suggesting that the insolubility was due to the reaction between glutaraldehyde and the amino groups of lysine, in this way introducing cross-links between the molecules.

The retention of only around 15% of the original enzymatic activity might perhaps be explained by the relatively smaller surface area which the polymerized molecules have available for contact with the solvent containing the substrate molecules. The insoluble enzyme particles represented a variety of particle sizes, as seen from a striation in the precipitates during the centrifugation used in the purification. Further experiments are required before uniform preparations of insoluble enzymes can be prepared by the glutaraldehyde method.

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Resistance of alkaline phosphatase of human serum to the protease of *Streptomyces griseus*

ARFORS, BECKMAN AND LUNDIN[1] described a genetically controlled variation of the alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) present in human blood serum. Type 1 phosphatase appears in starch-gel electrophoresis as a single zone of activity. The electrophoretic mobility of this enzyme is decreased after incubation with neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18)[2]. Type 2 phosphatase exhibits an additional zone of activity which is resistant to neuraminidase[2]. The evidence which indicates that the latter phosphatase component is derived from the intestine has been recently