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Coprecipitation of Enzymes with Water Soluble Starch—An Alternative to Freeze-drying

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Abstract—Krill proteases were prepared in solid form from a partially purified extract by coprecipitation of the enzymes with water-soluble starch in an organic solvent at 22°C. The precipitation did not affect the activity of the enzymes. The recovery of proteolytic activity was 100%. The thermostability of the krill proteases increased when incorporated in the starch precipitate. No reduction in enzymatic activity could be seen after storage at +50°C for 99 days. After milling the coprecipitate could be dispensed. The enzyme preparation consisted of irregular needle-shaped particles. This simple precipitation technique offers an alternative to freeze-drying or spray-drying.

The Antarctic Krill (*Euphausia superba*) has recently been shown to provide a potential source of proteolytic enzymes for wound cleansing or debridation (Hellgren et al 1986). From the crude krill extract, a fraction with high proteolytic activity is isolated. Trypsin and carboxypeptidase A and B activities have been identified in this fraction. The average molecular weight of the enzymes is approximately 30 000 daltons.

Preliminary in-vitro experiments have shown exceptionally good effect on necrotic tissue (Hellgren et al 1986). Clinical studies have been started to evaluate the potential wound cleansing effect. The formulation which is used in the clinical evaluation is a freeze-dried product. However, lyophilization is expensive, time-consuming and the residue cannot readily be dispensed. Therefore an alternative technique giving a solid product or powder with improved dispensing properties would be desirable.

The aim of this study was to establish whether coprecipitation with soluble starch in an organic solvent could replace lyophilization and whether the product obtained in this manner displayed superior handling properties and retained its activity after reconstitution.

Materials and Methods

Partially purified proteases from antartic krill were obtained from Pharmacia AS, Denmark. Carbonic anhydrase, *p*-nitrophenylacetate and bovine serum albumin were purchased from Sigma Chemical Co, USA. Tyrosine and casein substrate were purchased from Merck, West-Germany. Low molecular mass starch (MW = 12 700) and water soluble high molecular mass starch (MW = 100 000) were obtained from Reppe Glykos AB, Växjö, Sweden. Ethanol 99·5% was from AB Svensk Sprit, Sweden. Acetone, isopropanol and other chemicals were of analytical grade.

The activity of carbonic anhydrase was determined with the chromogenic substrate *p*-nitrophenylacetate (Pocker & Stone 1965). The assay mixture consisted of 4·7 mL 50 mM Tris-HCl buffer pH 7·4, 0·25 mL of substrate solution in absolute ethanol at the appropriate concentrations and 0·05 mL of enzyme solution. The hydrolysis was followed spectrophotometrically at 400 nm at room temperature (+22°C). The velocity of the reaction was measured as the initial increase in absorbance during 1 min. Correction was made for the spontaneous hydrolysis of the substrate in samples not containing the enzyme.

The assay of proteolytic activity was performed using denatured casein as a substrate and measuring the tyrosine and tryptophan content with a phenol reagent at 578 nm (Kunitz 1947). One unit releases one μmol of tyrosine min^{-1} at +35°C, pH 7·5.

The protein content was determined by the method of Lowry et al (1951). Ultrafiltration of proteases was performed with an Amicon Ultrafiltration cell equipped with a membrane having a cut-off of 10 000 daltons.

Precipitation of enzymes with starch

Krill proteases, or carbonic anhydrase, were diluted or dissolved in 3–4·5 mL Tris-HCl buffer pH 7·4 to give concentrations between 1 and 30 mg mL^{-1}. Low molecular mass starch MW 12 700 (LMM starch) or water-soluble high molecular mass starch MW 100 000 (HMM starch) were then added giving final concentrations of 15, 20 and 25% (w/w). The resulting aqueous phase was mixed with the organic phase (100 mL of acetone, ethanol or isopropanol) with stirring. The precipitation was rapid and the resulting coprecipitate was easily collected on a Munktell filter. The precipitation was carried out at room temperature. The precipitate was dried in a vacuum for 72 h at 35°C and further micronized by milling in a cutting mill (APEX Constructions London).

The density was measured by an air pychnometer from Beckman Instruments, USA. Surface area was estimated by a Fischer subsieve sizer from Fischer Scientific Company, USA. Sieving was performed following a standardized procedure with sieves from Veco, Eerdeek, Holland. Scanning electron microscopy was carried out as follows. The powder or particles were fixed with glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated and coated with gold/palladium. The specimens were examined and photographed in a Jeol electron microscope.

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Results

The results from the precipitation studies are summarized in Table 1. The maximal solubility of the LMM starch is approximately 15% (w/w). At that concentration a quantitative recovery of proteolytic activity was obtained in all three solvents. Lower concentrations of LMM starch reduced the yield considerably. The HMM starch in a 15% solution on the other hand, produced a precipitate which could not conveniently be collected on a filter and considerable losses in activity resulted, especially in acetone and ethanol. However, a quantitative recovery of proteolytic activity was obtained at a concentration around 25% in all solvents. The protein concentration had no influence on the recovery, at least at concentrations < 30 mg mL^{-1}.

Table 1. Some properties of krill proteases precipitated with different starches and solvent.

Starch	Solvent	Yield %[a]	Enzymatic units mg^{-1} solid[a]	Protein μg mg^{-1} solid[a]	Specific activity (units mg^{-1})
LMM MW 12 700					
15%	Acetone	99 ± 7	0·156 ± 0·005	101·4 ± 0·4	1·54
15%	Ethanol	107 ± 7	0·185 ± 0·005	117·4 ± 0·4	1·58
15%	Isopropanol	108 ± 3	0·164 ± 0·008	100·5 ± 1·2	1·63
HMM MW 100 000					
15%	Acetone	80	0·126	—	—
15%	Ethanol	46	0·139	—	—
15%	Isopropanol	103	0·169	—	—
25%	Ethanol	104 ± 10	0·101 ± 0·006	59·4 ± 0·3	1·70

[a] Mean ± s.d., n = 3

The initial proteolytic activity in the water phase was 30·6 ± 0·7 units mL^{-1} corresponding to 23·3 ± 2·3 mg protein mL^{-1}. The specific activity is approximately 1·3 units (mg protein)$^{-1}$. After precipitation the specific activity was 1·6–1·7 depending on the starch used, corresponding to a 1·3-fold purification.

Properties of the precipitate

The coprecipitation of krill proteases with starch after drying, yielded a readily water-soluble powder. The most soluble product was obtained with LMM starch. The dissolution rate in water was comparable to that for freeze-dried krill proteases. HMM starch gave a slightly less soluble product. The recovery of proteolytic activity was 100% when a sufficient amount of starch was used, Table 1. This indicates that no denaturation of the enzyme had taken place although the precipitation was carried out at +22°C.

The precipitate after drying consisted of a rather coarse powder with irregular particles or aggregates which needed to be further processed by milling or grinding to obtain a more uniform size distribution. The precipitate containing LMM starch was more difficult to mill owing to adhesion to the surface of the mill and also to formation of large agglomerates. As the precipitate from HMM starch did not have similar disadvantages, it was further characterized. Two batches, A and B, containing 15 and 30 g starch, respectively, were produced and characterized. Table 2 summarizes the technical properties. The density revealed that the precipitate was light and that the precipitates are, in that respect, reproducible. The surface area was relatively small and did not change pronouncedly with altering porosity.

The results from the particle sieve analysis are shown in Table 3. The major fraction was found between 90–710 μm with a peak around 180–380 μm. The shape of precipitated particles is shown in Fig. 1 revealing irregular needle-shaped particles.

Thus, the particles produced after the precipitation are irregular "needles" with low density giving a light powder with a non-free-flowing character. Initial dispensing experiments have revealed that the powder can be handled with acceptable accuracy within specified limits.

The thermal stability of the coprecipitate is excellent. No significant decrease in proteolytic activity could be detected after 99 days at +50°C. There was no significant difference

Table 2. Technical properties of HMM starch precipitate containing krill proteases.

Precipitate	Batch size	Density[a] g cm^{-3}	Porosity[a]	Surface[a] cm^2 g^{-1}
A	15 g	0·853 ± 0·005	0·675 ± 0·006	3095 ± 64
			0·575 ± 0·010	2952 ± 71
			0·466 ± 0·008	2834 ± 65
B	30 g	0·877 ± 0·003	0·634 ± 0·004	2473 ± 52
			0·557 ± 0·003	2393 ± 45
			0·471 ± 0·006	2332 ± 23

[a] All values are given in mean ± s.d. (n = 3).

Table 3. Size distribution of the precipitate in per cent by weight.

	Part by weight (%)	
Particle size μm	Batch A	Batch B
> 1000	2	1
710–1000	12	11
500–710	20	14
360–500	22	18
180–380	28	33
90–180	12	19
45–90	3	3
< 45	1	1

All measurements are made in duplicate. Mean values are given.

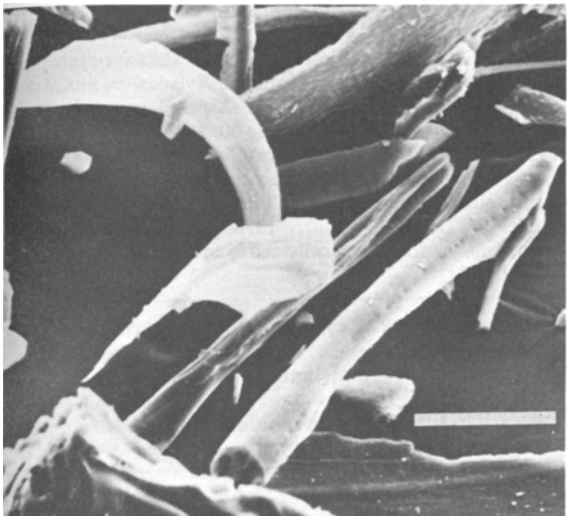

FIG. 1. Scanning electron micrograph of co-precipitated krill proteases with HMM starch. The bar indicates 50 μm.

Table 4. Thermostability of coprecipitate of krill proteases and starch.

Type of starch	Day no	Temp. C	Proteolytic activity u mg^{-1}	Activity remaining %
LMM MW 12 700	Initial	—	0·184	100
	7	25	0·186	101
	7	50	0·185	101
	28	25	0·189	103
	28	50	0·169	92
	63	25	0·192	104
	63	50	0·184	100
	99	25	0·185	101
	99	50	0·168	91
HMM MW 100 000	Initial	—	0·093	100
	7	25	0·101	109
	7	50	0·100	108
	28	25	0·096	103
	28	50	0·092	99
	63	25	0·104	112
	63	50	0·101	109
	99	25	0·099	106
	95	50	0·094	101

All measurements are made in duplicate. Mean values are given.

in stability between coprecipitates prepared from the two different starches. Table 4 summarizes the results.

Carbonic anhydrase was used to study the effect of precipitation on the enzymatic characteristics. The enzyme was chosen as a model enzyme because the molecular weight is approximately the same as that for the krill proteases. Furthermore, the enzyme is commercially available in a pure form. The enzymatic yield was quantitative and the K_m of the native enzyme and the reconstituted precipitated enzyme were similar (Km ca 20 mM). This result indicates that there is no interference between the starch and the substrate/enzyme.

Discussion

The term coprecipitation is used to denote the occlusion by incorporation of foreign substances during the formation of a precipitate (Kolthoff 1932). In the present paper we have used coprecipitation to advantage for the preparation of an enzyme powder from a partially purified extract.

Krill proteases could be coprecipitated at +22°C with a water-soluble starch in an organic solvent, e.g. acetone, ethanol and isopropanol with quantitative recovery of activity. The incoporation of krill proteases into a coprecipitate also increased their thermostability. This stabilization is probably attributable to the interaction of enzyme molecules with the starch support. In principle, protein molecules can form hydrogen bonds with hydroxy groups of the support. We have earlier investigated the thermal stability of pure freeze-dried krill proteases in 30 mM Tris-HCl buffer pH 7·5. After 50 days at +50°C, a 25% decrease of proteolytic activity was observed. Coprecipitated krill proteases, however, do not show any significant decline in proteolytic activity after storage under similar conditions.

This precipitation step also seemed to effect further purification of the enzyme extract which is reflected in an increase in the specific activity. A plausible explanation could be that low-molecular compounds like aminoacids and small peptides are not precipitated together with starch. Another factor which may contribute is an interaction between the polysaccharide and the enzyme to give a more active form of the enzyme. This phenomenon has been reported recently by Panda et al (1987) with Xylanase together with a polysaccharide consisting of glucose monomeric units.

No significant difference between the kinetic constant K_m of the coprecipitated carbonic anhydrase and the native form was found. However, it is necessary that several other enzymes are tested before a more general conclusion can be drawn that coprecipitation of enzymes with starch in an organic solvent does not effect the kinetic parameters. From the literature it is documented that acetone precipitation has been used for the isolation of ribosomal protein which retained normal physiological properties (Barritault et al 1976).

Precipitation of enzymes in an organic solvent often leads to a low recovery of enzymatic activity and difficulties in processing. The use of coprecipitation with starch reduces the denaturation of the protein by physically entrapping of the enzyme into the support matrix thereby protecting the enzyme from the denaturing environment. The method gives a powder that can be further processed or be filled in vials. It may also be used directly for topical applications.

Schröder (1984) used an emulsion technique for preparing precipitated protein containing starch microspheres. In his method, the carbohydrate was dissolved together with the protein to be entrapped in buffer and thereafter emulsified in cotton seed oil. The emulsion was then poured into acetone whereby the microspheres precipitated. The release profile of entrapped proteins from these spheres was followed in-vitro.

The coprecipitation technique described in this paper effects a stabilizing effect on the enzymes and yields irregular particles which release the enzymes immediately when water is added. The crystallized spheres, on the other hand, give sustained release profiles of entrapped proteins.

To summarize, coprecipitation of enzymes with starch constitutes an interesting method for preparing enzymes in solid form from solution that may in many cases, replace techniques like freeze-drying and spray-drying.

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