IMMOBILIZATION OF CHYMOTRYPSIN (E.C.3.4.21.1), SUBTILISIN (E.C.3.4.21.14) AND NEUTRAL PROTEINASE FROM *Bacillus subtilis* BY COVALENT BINDING TO BENZOQUINONE-ACTIVATED PEARL CELLULOSE

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Chymotrypsin (E.C.3.4.21.1), subtilisin (E.C.3.4.21.14), and a neutral proteinase from *Bacillus subtilis* were immobilized by covalent binding to benzoquinone-activated pearl cellulose. The yield of the immobilized protein was 55% in the case of chymotrypsin and 50% in the case of subtilisin and neutral proteinase from *B. subtilis*. The determination of activation energy at a low substrate concentration showed that the enzyme activity is limited by diffusion under these conditions. The activity yields are generally very good yet the activity of the enzymes immobilized is relatively low, most likely because of the presence of benzoquinone, as shown in experiments with the immobilization of chymotrypsin by the same technique on supports with different hydrophilicity.

Immobilized enzymes have received application as efficient catalysts in pharmaceutical industry[1,2], food technology[3-6], analytical chemistry[7-9] and organic synthesis[10-14]. One of the hurdles in the path to an even more wide-spread use of immobilized enzymes is the relatively high price of appropriate supports and the inconvenient methods of attachment which employ either highly toxic (cyanogen bromide) or otherwise dangerous reagents (epichlorohydrin, divinyl sulfone and others) or coupling agents obtainable in larger quantities with difficulties (such as water-soluble carbodiimides, the Woodward reagent K, *etc.*). An immobilization method prospective from the viewpoint of industrial applications is the benzoquinone coupling, especially when applied to pearl cellulose which because of its structure, porosity, and other features is especially convenient for immobilization and moreover is commercially available in large quantities at a relatively low price.

This paper reports on the immobilization of three enzymes representing one of the basic proteinase types of chymotrypsin, subtilisin, and a neutral proteinase from *Bacillus subtilis*, by covalent coupling to benzoquinone-activated cellulose.

EXPERIMENTAL

Chymotrypsin was a commercial product of Spofa, Prague; its specific activity was 4·61 c.u./mg protein and the protein content was 794 mg/g preparation. Subtilisin was a product of NOVO

Industri (Denmark); its activity was 22·16 c.u./mg protein and the protein content was 116 mg/g preparation. The neutral proteinase from *Bacillus subtilis* was purchased from Pharmachim (Bulgaria); the activity of the preparation bearing the commercial designation E-30 was 4·92 c.u./mg protein, the protein content being 196 mg/g preparation. Subtilisin and the neutral bacterial proteinase were dialyzed prior to immobilization 24 h against tap water to remove salts. Pearl cellulose was a product of Severočeské chemické závody, Ústí nad Labem, and had the following characteristics: water regain (pH 8·0) 7·06 g water/g, mean diameter of swollen beads 0·42 mm, proportionality constant $K = 10·6$ (ref.[15]), and sedimentation rate 0·28 cm/s. Benzoquinone was prepared by sodium perchromate oxidation of hydroquinone[16].

Protein concentration was measured according to Hartree[17] and the proteolytic activity by the method of Slavík and Smetana[18] and expressed in casein units (c.u.). One casein unit is defined as the quantity of enzyme which in 1 min will generate from 2% casein solution at 30°C and optimal pH 1 mg of fragments soluble in 0·1 mol l^{-1} trichloroacetic acid. The activation of the support was carried out by the conventional procedure[19] as well as the coupling of the enzyme; the protein concentration was varied during the coupling. The immobilized enzyme was stored as a suspension in 0·2M phosphate buffer at pH 7·8 and 0–5°C.

For comparison purposes chymotrypsin was coupled by the same method also to aminoethyl cellulose (Whatman AE-50) and aminopolystyrene, prepared by nitration of polystyrene and reduction of the nitro groups[20]. The support, the binding, and the immobilized enzyme were characterized by methods recommended for uniform characterization of immobilized enzymes[21].

RESULTS AND DISCUSSION

The quantity of protein immobilized on 1 g of support depends as a rule on the concentration of the protein in solution, *i.e.* on the quantity of enzyme used provided that the quantity of the support and the final volume of the suspension do not change. We have examined this dependence with all three enzymes. The protein-binding yield expressed in per cent according to[21] was independent (except for the lowest concentrations) within experimental error of the quantity of the native enzyme used. This shows that the binding capacity of the activated support was not exhausted even when the largest quantities of the native enzyme were used. The binding yield was 55% with chymotrypsin and 50% with the remaining two enzymes. The deviations observed at lower concentrations of the native enzyme can be accounted for by the low sensitivity of the analytical method at these concentrations; these values were not therefore included in the final evaluation. The profile of the dependence is shown in Fig. 1.

The specific activity of the immobilized enzyme at different concentrations of the protein immobilized (the so-called E-test[22]) can serve as a criterion of the effect of diffusion under the experimental conditions given. We determined therefore with all immobilized enzymes this effect at a standard concentration of the substrate (2% casein). The plot of activity of all three enzymes as a function of the concentration of immobilized protein is shown in Fig. 2. The specific activity was independent of the concentration of the proteins immobilized over the entire concentra-

tion range examined. This may indicate the absence of the diffusion effect. Since, however, the substrate concentration was relatively high (higher than the K_m-value) and since this analytical method is not sensitive enough to reveal minor diffusion effects, we used a more sensitive criterion of the diffusion effect, namely the determination of the activation energy of the enzymatic reaction as a function of the substrate concentration. We observed that the rate of the enzymatic reaction is affected by diffusion in all cases at low substrate concentration or high temperature (Fig. 3).

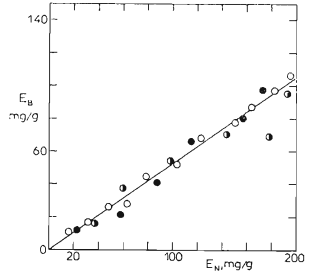

FIG. 1

Quantity of proteins immobilized as function of quantity of native proteins used ○ chymotrypsin, ◑ proteinase from *B. subtilis*, ● subtilisin

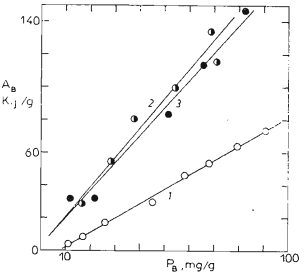

FIG. 2

Activity of immobilized enzyme as function of quantity of proteins immobilized. *1* chymotrypsin, *2* proteinase from *B. subtilis*, *3* subtilisin

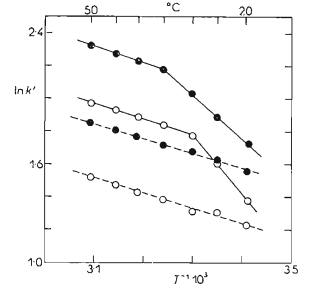

FIG. 3

Arrhenius function at different concentrations of substrate (casein) ——— 2%, – – – – 4%. ○ Chymotrypsin, ● subtilisin

A criterion of importance from the viewpoint of practical applicability of immobilized enzymes is their operational stability. The latter was determined with immobilized chymotrypsin, subtilisin, and the neutral proteinase from *B. subtilis* in a column reactor by the procedure described elsewhere[23,24]. The operational stability of these immobilized enzymes in continuous operation at 30°C is relatively low (Table I). This shows that the enzymes immobilized on this supports by the procedure described can be used at low temperature only. The destabilization can be caused both by the effect of the support (this is extremely unlikely with pearl cellulose) or by the mode of binding. To eliminate the unfavorable effect (if any) of the support we immobilized chymotrypsin by the same procedure (*a*) on aminopolystyrene where a destabilizing effect of the support may be expected to result from the hydrophobic character of the support backbone, and (*b*) on aminoethylcellulose, which is more hydrophilic than pearl cellulose because of the presence of the substituent. The operational stability was low in both cases and was comparable with the stability of the same enzyme immobilized on pearl cellulose (the halflife of inactivation of chymotrypsin

TABLE I
Characteristics of immobilized enzymes

Quality	Chymotrypsin	Subtilisin	Neutral proteinase from *B. subtilis*
Specific activity of native enzyme (c.u./mg protein)	4·61	22·16	4·92
Protein content of preparation of native enzyme, mg/g	794	116	196
Protein content of immobilized enzyme mg/g	59	44	47
Native proteins for binding mg/g support	108	88	98
Binding yield, %	55	50	50
Activity of immobilized enzyme c.u./g	47	73	89
Activation energy (kJ/mol), $S = 0{\cdot}4\%$ $S = 2\%$	16·03 53·63	16·22 56·42	— —
Operational stability $t_{1/2}$, h	12·2	18·2	14·8

immobilized on aminopolystyrene and aminoethylcellulose was 10·6 and 6·4 h, respectively. These results indicate that the immobilization method using benzoquinone has a destabilizing effect on the proteinases immobilized This increase of enzyme inactivation during continuous operation is most likely due to the highly hydrophobic character of benzoquinone used as the binding reagent and to its proximity to the peptide chain of the enzyme. The effect could then be analogous to the destabilizing effect of apolar solvents.

Table I shows the complete characteristics of the enzymes immobilized; the properties of the support are described in full under Experimental and are therefore not included in the Table.

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