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INVESTIGATION OF SOME α-AMYLASES BY HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

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SUMMARY

α-Amylases of different origins were investigated with a Synchropak propyl column and a linearly descending salt gradient in order to isolate the active fraction from among the inactive proteins present. Fractions collected by several injections were assayed for enzymatic activity by the modified Wohlgemut method. An experimental hydroxypropyl packing was tested for the separation of α-amylases under isocratic conditions, using a dilute buffer or plain water as eluent. The chromatograms obtained can serve as fingerprints for the quality control of some industrial proteins.

INTRODUCTION

Hydrophobic-interaction chromatography (HIC) was first developed in the early 1970s, using agarose gels with bonded alkylamine ligands[1–3]. High-performance HIC is a relatively new addition to the rapidly advancing methods of protein separation.

The retention mechanism is based essentially on the same forces (London-type dispersion) as in reversed-phase chromatography. In both instances hydrophobic functional groups are bound to the substrate and used as the stationary phase. However, in reversed-phase packings the functional groups are very densely distributed, producing a strong hydrophobic interaction between the proteins and the stationary phase. As a result, organic solvents must be used to elute the proteins, which then lose some of their activity.

By contrast, the functional groups in hydrophobic-interaction packing materials are much more sparsely distributed, short, hydrophobic ligands, so that elution can be accomplished by buffers without appreciably denaturing the proteins. This is generally achieved by using reverse gradients in which the sample is eluted by going from high to low salt concentrations. Because proteins retain their structures under such conditions, it is especially suitable for studies where enzymatic activity is of prime importance.

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Melander and Horváth[4,5] have given an explanation for the role of neutral salts in the hydrophobic interaction of proteins, based on the solvophobic theory of Sinanoglu and co-workers[6,7]. According to their treatment, the effect of salt type on protein retention can be related to the molal surface tension increment of the salt[4]. Thus, salts with higher molal surface tension increments produce increased retention at equal molal salt concentrations. The magnitude of the effect depends also on the properties of the protein and the stationary phase. The effect of the stationary phase and the operational variables[8–19] have been extensively investigated. Separation and investigation of α-amylases by HIC have also been reported[9,20].

In our laboratory, hydrophobic-interaction HPLC has been used to study a series of industrial enzymes such as α-amylases, glycoamylases and glycooxidases. In this paper, we report some results obtained in the investigation of α-amylases. The goal of this investigation was to isolate the active fraction from among the inactive proteins present in enzyme preparations of different origins.

EXPERIMENTAL

Materials

The investigation was carried out on a Merck–Hitachi HPLC system consisting of a Model 655A-11 pump, a Model L-5000 low-pressure gradient system, a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector valve with a 20-μl loop and a Model 655A-22 variable-wavelength UV detector. Chromatograms were recorded and evaluated with a Merck–Hitachi Model D-2000 chromato-integrator.

Separations were performed on a SynChropak propyl (particle size 6.5 μm) column (250 x 4.1 mm I.D.), obtained from SynChrom (Linden, IN, U.S.A.) and an experimental hydroxypropyl (particle size 10 μm) column (250 x 4.6 mm I.D.) developed by Dr. R. Ohmacht at the University Medical School, Pécs, Hungary.

Protein standards (ribonuclease A, ovalbumin, chymotrypsin, chymotrypsinogen A) and α-amylase (Type II A, bacterial) were obtained from Sigma (St. Louis, MO, U.S.A.), Brew-n-zyme AT, a thermostable bacterial α-amylase, from Jan Dekker BV, Naarden International (Wormerveer, The Netherlands) and Termamyl 60 L thermostable α-amylase from Novo (Denmark).

Analytical-reagent-grade ammonium sulphate and potassium dihydrogenphosphate were obtained from Reanal (Budapest, Hungary). Distilled water was prepared by double distillation in glass.

Methods

On the SynChropak propyl column, a descending linear salt gradient was used from 1.0 to 0.1 *M* ammonium sulphate in 0.02 *M* potassium dihydrogenphosphate buffer at pH 7.0. The flow-rate was 1 ml/min, and detection was accomplished at 280 nm. On the experimental hydroxypropyl column, isocratic elution was carried out by using 0.1 *M* ammonium sulphate in 0.02 *M* potassium dihydrogenphosphate buffer at pH 7.0 or plain distilled water containing 20 ppm of Ca^{2+}.

Samples were prepared by dissolving about 10 mg of α-amylase in 1 ml distilled water. The industrial enzyme samples were diluted with distilled water in a 1:9 ratio. Each sample was chromatographed at least in triplicate.

Determination of enzymatic activity

The enzymatic activity of α-amylase was determined by the modified Wohlgemut method[21] (Naarden Assay No. 2-124). This assay is based on determining the time required to hydrolyse starch to dextrin of a definite size, as indicated by the colour of the dextrin–iodine complex. This colour is compared with a colour standard of glass disks (Hellige No. 620 S-5).

RESULTS AND DISCUSSION

Before injection of the α-amylase samples, the SynChropak propyl column was tested by injection of some protein standards generally used for the evaluation of HIC columns. First, a 30-min linear gradient was used in accordance with the test chromatogram published. In order to shorten the analysis time and improve peak sharpness, a steeper 20-min linear gradient was also applied, as shown in Fig. 1. Since with the 20-min gradient the retention times decreased and the peaks became sharper without a significant decrease in resolution, the enzyme samples were investigated under these conditions.

Fig. 2 shows the chromatogram of the Sigma α-amylase standard. After the first chromatogram, sample injection was repeated five times and the eluent fractions corresponding to the individual peaks were pooled. In this instance, only two fractions were collected. Enzymatic activity in the collected fractions was determined by the modified Wohlgemut method. The enzyme activity measured is indicated in Fig. 2 by the dashed line.

In Fig. 3 the chromatogram of Brew-n-Zyme AT is shown. Again, the separated fractions from five chromatograms were pooled. In this instance, three fractions were collected and tested for enzyme activity. The dashed line shows the enzyme activity of the fractions.

Fig. 4 shows the chromatogram of Termamyl 60 L. Three separate fractions

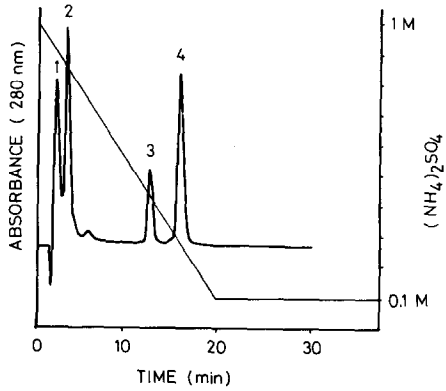

Fig. 1. Separation of a standard protein mixture by HIC. Column, SynChropak propyl; mobile phase A, 1 *M* $(NH_4)_2SO_4$–0.02 *M* KH_2PO_4 (pH 7.0); mobile phase B, 0.1 *M* $(NH_4)_2SO_4$–0.02 *M* KH_2PO_4 (pH 7.0); 20-min linear gradient from A to B; flow-rate, 1 ml/min. Standards: 1 = ribonuclease A; 2 = ovalbumin; 3 = α-chymotrypsin; 4 = chymotrypsinogen A.

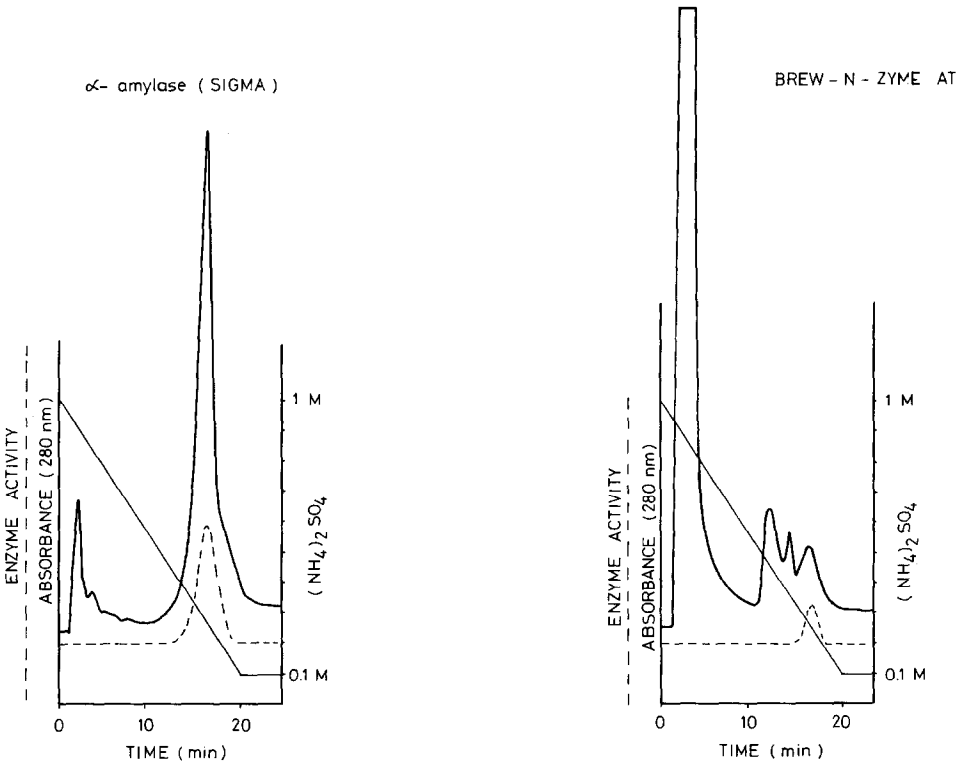

Fig. 2. Chromatogram of α-amylase (Sigma). Conditions as in Fig. 1.

Fig. 3. Chromatogram of Brew-n-zyme AT industrial enzyme. Conditions as in Fig. 1.

were collected and tested for enzyme activity. Again, the fraction eluted between 16 and 18 min contains the activity, as shown by the dashed line.

Fig. 2 shows that in the purified Sigma product almost all proteins present are responsible for the enzymatic activity. In contrast, in the industrial enzyme preparations a large amount of other proteins can also be found. For instance, in the Brew-n-Zyme chromatogram (Fig. 3), there are other proteins in the vicinity of the active protein, which may represent other types of enzymatic activity. The chromatograms shown demonstrate at the same time that HIC can also be efficiently used for preparative purposes, producing enzyme preparations of high purity and high specific activity.

Next, some preliminary results will be reported on the use of the experimental hydroxypropyl packing. This packing shows an intermediate retention capacity between reversed-phase and HIC columns. A test mixture containing dimethyl, dipropyl and dibutyl phthalate was used to compare the hydrophobicity of the two columns investigated. With methanol–water (1:1) as the mobile phase, the hydroxypropyl column provided a well resolved chromatogram with symmetrical peaks and retention times of 5.6, 10.0 and 16.1 min, respectively. Under the same conditions the Syn-Chropack propyl column furnished two overlapped peaks at 3–3.6 min. On this column a much weaker mobile phase, methanol–water (1:9), provided a similar sep-

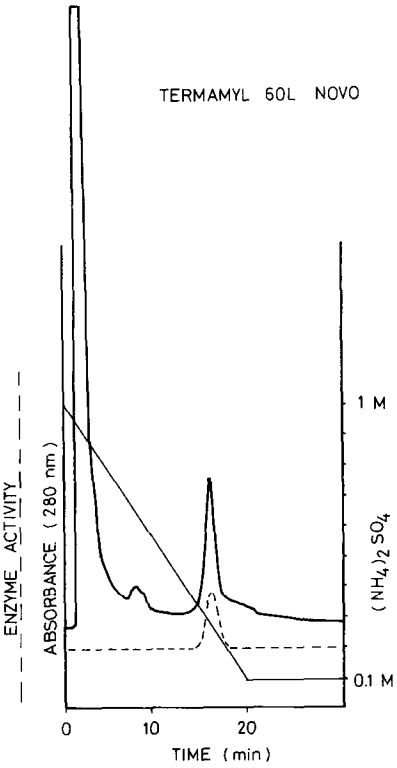

Fig. 4. Chromatogram of Termamyl 60 L industrial enzyme. Conditions as in Fig. 1.

aration of the test mixture to that obtained on the hydroxypropyl column (retention times 4.6, 7.6 and 14.2 min, respectively).

The retention of proteins on the hydroxypropyl column at high salt concentrations was very strong, resulting in long retention times and broad peaks under HIC conditions. For this reason we tried to use this column under isocratic conditions, instead of the reverse gradient used for HIC columns.

First, we compared the performance of the column with buffer B of the previous buffer system (0.1 *M* ammonium sulphate in 0.02 *M* potassium dihydrogenphosphate) or distilled water containing 20 ppm of Ca^{2+} in the form of calcium acetate. According to the literature[21,22], metal ions have been used to protect α-amylase activity against pH, temperature and proteases. Calcium has been found the most effective metal ion in preserving the activity of the enzyme.

With both eluents we obtained chromatograms containing a large number of completely or partially resolved peaks. However, the chromatograms obtained with water as eluent were superior, the peaks being better resolved than in the buffer system. In Fig. 5, the chromatogram of the Sigma α-amylase shows that, in contrast to the two large peaks obtained by HIC, a large number of peaks (about 10–12) can be distinguished in the chromatogram. In Fig. 6 the chromatogram of Brew-n-zyme and in Fig. 7 the chromatogram of Termamyl 60 L are shown. There are a large number of peaks of different proportions and partly at different retention times.

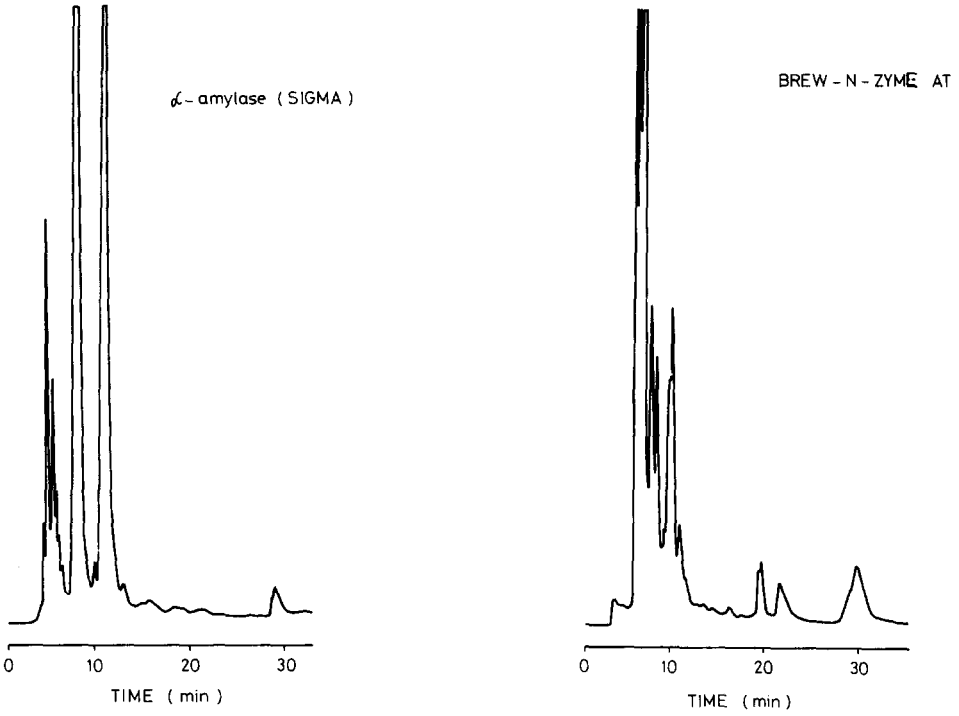

Fig. 5. Chromatogram of α-amylase (Sigma). Column, hydroxypropyl (experimental); mobile phase, distilled water (20 ppm Ca^{2+}); flow-rate, 1 ml/min (isocratic).

Fig. 6. Chromatogram of Brew-n-zyme AT industrial enzyme. Conditions as in Fig. 5.

Culture filtrates of *Bacillus subtilis* are the richest sources of α-amylase and different proteases, and the industrial enzyme preparations are more or less purified culture filtrates. For this reason, several other proteins of different molecular weights, even peptides, may be present in the enzyme preparations[21,23].

The chromatograms obtained under isocratic conditions and using plain water or very dilute buffers as eluent were highly reproducible and can serve as fingerprints for the characterization of some industrial protein products.

CONCLUSIONS

High-performance HIC can be used for the comparison and characterization of industrial enzymes of different origin. In addition, HIC can be efficiently used for preparative purposes, producing enzyme preparations of high purity and high specific activity.

Packings with intermediate retention capacity (hydrophobic character) between reversed-phase and HIC columns seem to possess a high resolving power for certain proteins under isocratic elution conditions and can be used for the quality control of some industrial protein products.

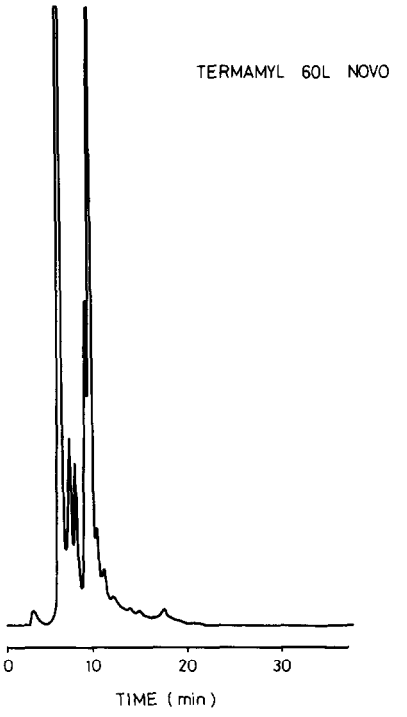

Fig. 7. Chromatogram of Termamyl 60 L industrial enzyme. Conditions as in Fig. 5.

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