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Review

Chromatographic methods for amylases

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Abstract

This review surveys recent developments in chromatographic methods for the separation of amylases from complex extracts, including the separation of isozymes. It contains two tables with the properties and molecular characteristics of α- and β-amylases from different sources as well as an updated review of methods for the determination of amylase activity. The main subject of this review is a detailed evaluation of the application of newly developed chromatographic methods for the purification of amylases.

Keywords: Reviews; Amylases; Enzymes

Contents

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List of abbreviations

α-CD	α-Cyclodextrin (cyclohexaamylose)
β-CD	β-Cyclodextrin (cyclohexaamylose)
EDTA	Ethylenediamine-tetraacetatic acid
CM	Carboxymethyl-
DEAE	Diethylaminoethyl-
FG5P	*p*-Nitrophenyl O-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-O-α-D-glucopyranosyl-(1-4)-O-α-D-glucopyranosyl-(1-4)-O-α-D-glucopyranosyl-(1-4)-α-glucopyranoside
H-gel	Hydrophobic gel
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IMAC	Immobilized metal-ion affinity chromatography
MW	Molecular mass
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PNP	*p*-Nitrophenol
PyS-gel	Thiopyridine gel
SDS–PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
T-gel	Thiophilic gel
TIC	Thiophilic interaction chromatography

1. Introduction

1.1. Sources and distribution of amylases: their most relevant molecular and physicochemical properties

Amylases are enzymes catalyzing the hydrolysis of glycosidic α-1,4-bonds in polysaccharides such as starch and glycogen. Starch consists of a mixture of amylose (15–30%) and amylopectin (70–85%). Amylose is a linear polymer of glucose units linked by α-1,4 glycosydic bonds and amylopectin, a branched polymer containing α-1,6 branch points every 24–30 glucose residues [1].

α-Amylase hydrolyses the internal α-1,4 glycosidic links in amylose and amylopectin at random to produce a less viscous solution with lower MW products limited by the α-1,6 glycosidic bonds which form the branch points in the native starch (amylopectin) molecule. The products of hydrolysis have an α-configuration at the reducing glucose end [2]. α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1.) are calcium containing enzymes, with a single polypeptide chain folded into three domains belonging to the $(\beta/\alpha)_8$-barrel protein family [3]. The active site consists of aminoacid residues in loops projecting outwards from the C-terminal end of the barrel. In the α-amylase subfamily of the $(\beta/\alpha)_8$-barrel proteins, the third barrel forms a small separate, Ca^{2+}-stabilized structural domain participating in substrate binding [4].

α-Amylases are found in animal organs such as pancreas (human, pig, dog, horse) and salivary glands (human, pig, rat, etc.). However, the salivary secretions of horses, dogs and cats lack this enzyme [2]. They are also found in human plasma and urine but are not present in liver, nor in leukocytes. α-Amylase is found in higher plants, specially in germinated seeds of cereals such as malted barley, oats and wheat and in micro-organisms such as moulds (*Aspergillus oryzae*) and bacteria (*Bacillus subtilis*, var. *amyloliquefaciens*, *B. mesentericus*, *B. polymixa*, etc). α-Amylase is one of the key enzymes involved in carbohydrate digestion and metabolism in insects [5].

β-Amylases (α-1,4-glucan maltohydrolases, EC 3.2.1.2.) are exoamylases that hydrolyze starch beginning at the nonreducing ends, producing β-maltose and β-limit dextrins. They are present in some species of higher plants, particularly in seeds and germinated seeds of cereals (barley, rye, oats, wheat, sorghum, rice), and in soybean, maize and sweet potato tubers. The occurrence of β-amylases has also been reported in kiwi fruit [6], tap roots of alfalfa [7], tubers of taro [8] and mustard seedlings [9]. β-Amylases are also found in bacteria such as *Bacillus megaterium*, *B. circulans*, *B. cereus*, *B. polymixa*, *Pseudomonas* and *Streptomyces sp.* [1]. In higher plants, β-amylase molecule has been characterized mainly using enzymes purified from organs enriched in starch such as sweet potato tubers, seeds of various cereal species such as barley, wheat, rice and other higher plants such as soybean [9]. In these starch enriched organs, β-amylase may play a role in the mobilization of starch during seed germination or

the sprouting of tubers. In contrast, relatively little information is available on the purification and molecular characterization of β-amylase from the green leaves of higher plants. The characterization of β-amylases from *Vicia faba* leaves has been reported [9] In mustard (*Sinapis alba*) seedlings, β-amylase is the sole amylolytic enzyme, as these seedlings characteristically lack α-amylases. Furthermore, while β-amylase is activated from a pre-existing zymogen form during the germination of cereal seeds, in mustard seedlings it is synthesized de novo in the cotyledons under the influence of light. Several features of purified mustard β-amylase distinguish it from β-amylases from storage organs. For instance, it is not susceptible to inhibition by cyclodextrins which are competitive inhibitors of β-amylases. An antiserum raised against mustard β-amylase did not cross-react with β-amylase from sweet potato tubers and barley seeds, indicating that mustard cotyledons enzyme is immunologically distinct from β-amylases from storage organs [9]. All β-amylases reported so far are monomeric proteins except for sweet potato β-amylase which is reported to be a homotetramer [10,11].

The specific action of α- and β-amylases on starch and its components depends on the presence of metal ions, pH, and temperature. This dependence differs markedly according to the origin of the enzymes. α-Amylases from higher plants are generally very stable in the pH range 5.5–8.0. Optimal pH for cereal α-amylase activity is between 4.5 and 6.0; irreversible inactivation occurs below pH 4.5 [12] (Table 1). Optimal pH for β-amylase activity is generally in the range 4.0–6.0; these enzymes are relatively stable in the acidic range above pH 3.6 [13] (Table 2). Although several of the plant enzymes are inhibited by sodium *p*-chloromercuribenzoate, this deactivation is slow and is dependent on the concentration of calcium ions present. It is believed that sulphydryl groups are not directly involved in amylolytic activity; the inhibition may be caused by a deformation of the molecular structure of the enzymes [12]. α-Amylases are inactivated by calcium chelating agents such as EDTA. Excess of

Table 1
Properties of α-amylases

Source	MW ($\times 10^{-3}$)	Optimal pH	Optimal temperature (°C)	Stability pH range	Ref.
Barley		5.5	47.5	5.5–8.0	[12]
Barley (malted)	45 and 59	4.7–5.5	48–55	5.0–8.0	[12]
Broad bean	45	5.6	45	5.5–8.0	[12]
Oats	45	4.7	47	5.0–7.5	[12]
Rye	45	5.0	47	5.0–7.5	[12]
Sorghum (malted)	50	4.5–5.0	>40		[12]
Soybean	45	6.0	55	5.5–8.0	[12]
Wheat	45	5.0	49	5.0–7.5	[12]
Kiwi		6.0	50		[6]
A. mylita[a]	58	9.5			[5]
Hog pancreas	45	6.9[b]		7.0–8.5	[12]
Human pancreas		6.9[b]		4.8–11.0	[12]
Human saliva	55	6.9[b]		4.8–11.0	[12]
B. subtillis		5.5–6.0	70		[15]
B. licheniformis		7.0	90		[15]
B. amylo-liquefaciens	58	5.5	50–70	6.0–12.0	[15]
B. circulans	45	7.0	50	6.0–9.0	[15]
A. niger	58	4.0–5.0		2.2–7.0	[15]
A. niger	61	5.0–6.0		5.0–8.5	[15]
A. oryzae		5.0	40	6.0–8.0	[15]
Chalara paradoxa	80	5.5	45	5.5–7.5	[63]

[a] *Antheraea mylitta* (Indian tasar silkworm).
[b] Chloride-activated enzyme.

Table 2
Properties of β-amylases

Source	MW ($\times 10^{-3}$)	Optimal pH	Optimal temperature (°C)	Stability pH range	pI	Ref.
Sweet potato	197	4.5–5.0	60	5.0–8.0	4.77	[62]
Soybean	61.7	6.0	45	5.0–10.0	5.0–5.9	[62]
Wheat	64.2	5.2		4.5–9.2	6.0	[62]
Malt		5.2		4.5–8.0	6.0	[12]
Mustard	58	5.8–6.2		3.0–8.0	4.6–4.8	[9]
Pea	56	6.0			4.35	[123]
Barley	56	5.2	>50	4.5–8.0	6.0	[62]
Ichoimo	60	6.0	55	4.3–8.5	5.0–5.2	[62]
Sorghum	20	5.0–5.5	30–40	4.3–8.5		[23]
Taro tubers[a]	66 and 67	5.0	60			[8]
Maize	65				4.40	[121]
Kiwi		6.0	35			[6]
B. megaterium No. 32	67	6.5		5.0–7.5	9.1	[15]
B. polymxa NCIB 8158	67	6.8	37	6.4–7.2		[15]
B. polymixa N° 72	44	7.0–8.0	45	4.0–9.0	8.35	[15]
B. cereus (var. mycoides)	35	7.0	50	5.0–10.0		[15]
Pseudomonas sp BQ06	37	6.5–7.4	45–55	6.5–8.0		[15]

[a] Two forms.

calcium ions reverses the inhibition caused by EDTA. In the absence of chelating agents, α-amylases contain enough bound calcium ions for full activity and the addition of excess calcium does not produce an increase in activity but favours maximal enzyme stability [12]. Calcium ions increase the stability of α-amylases from malt, pancreas and other sources, but decrease that of β-amylases from barley. Certain salt ions such as iodides (possibly through the liberation of traces of iodine), fluorides and heavy metals, irreversibly inactivate amylases. Reversible inactivation of β-amylase is caused by some oxidants such as peroxides and iodine [14].

1.2. Industrial and biotechnological applications of amylases

There are numerous industrial and biotechnological applications of α-amylases. Probably the largest volume is used for thinning starch in the liquefaction process in the sugar, alcohol, and brewing industries. α-Amylases are used in desizing of fabrics, in the baking industry, in the production of adhesives, pharmaceuticals, detergents, in sewage treatment, and in animal feed [15]. Bacterial α-amylases have two large fields of application [16]. The oldest is desizing, for which amylase is used to remove starch sizes from the warp of cotton fabrics. The most important use is starch liquefaction in preparation of glucose or fructose/glucose syrups. Prior to extensive hydrolysis, the starch must be solubilized. Usually the starch suspensions contain around 30% dry solids and form very viscous suspensions. In this application the B. licheniformis enzyme has proved superior to the *B. amyloliquefaciens* enzyme due to better heat stability. The *B. licheniformis* enzyme produces mainly maltose, maltotriose and maltopentaose with the maltohexaose formed initially being completely hydrolysed. By contrast, the *B. amyloliquefaciens* amylase produces maltohexaose as the major product.

Liquefaction of starch raw materials for production of alcohol is another important application. Bacterial α-amylases have almost completely superseded the traditional malt in this process. The brewing industry also employs α-amylase with advantage in the liquefaction of various starch raw materials that are used as adjuncts. α-Amylase also finds application in sugar cane processing, since small quantities of starch occur in sugar cane, and thus in sugar juice.

Fungal α-amylases such as *Aspergillus oryzae*

amylase are more saccharifying, i.e., produce more sugars than do the equivalent *Bacillus* enzymes. It is possible to obtain over 50% of maltose when starch is hydrolyzed by this enzyme. Syrups of high maltose concentration are useful for a number of purposes where special functional properties are required. Another important application of *A. oryzae* amylase is in the baking industry, where the enzyme is added to flour with a low amylase content. The amylase degrades starch in the dough, and the maltose formed serves as substrate for the baker's yeast during leavening. The low temperature stability of the *A. oryzae* amylase is important as it prevents extensive degradation of the crumb during baking [16]. One of the main biotechnological application of β-amylase is in the production of maltose syrups from solubilized starch, to be used in the food and pharmaceutical industries. To increase the maltose yield and to reduce the amount of branched oligosaccharides, β-amylase is used in combination with pullulanase (debranching enzyme). Thus, the technological importance of β-amylase is highest in the brewing, distilling, and baking industries, were starch is converted into fermentable sugars.

In clinical analysis the measurement of total serum amylase concentration is an important parameter which is increased in a number of acute lesions in the intestine, including perforated ulcers, mesenteric ischemia, strangulation or obstruction and appendicitis. The absence of salivary-type isozyme in intestinal contents has been confirmed [17]. Therefore, measurement of serum amylase isozymes will not distinguish between an intestinal disorder, in which the amylase from the intestinal lumen is reabsorbed into the circulation, and acute pancreatitis.

1.3. Classical methods for the isolation of amylases

Different techniques have been developed for the isolation and purification of proteins based on their properties, prior to their characterization or use in biotechnological and industrial processes. These methods may be based on differences in: (i) solubility, (ii) non-specific adsorption-desorption processes on inorganic supports, (iii) electrical charges as a function of pH (ion-exchange chromatography, preparative electrophoresis), and (iv) molecular size (gel-permeation chromatography). These methods will be referred to as "classical methods", as opposed to those based on more specific interactions. The dependence of the solubility of proteins on solvent composition very often constitutes the starting point for a purification process. Salting out techniques, precipitation with cold water-miscible organic solvents, precipitation with organic polymers and isoelectric precipitation constitute good examples of this principle [18,19]. Non-specific adsorption of proteins on to certain inorganic materials such as calcium phosphate (hydroxyapatite), alumina gels and diatomaceous earths, followed by subsequent protein desorption have also been widely applied as purification procedures [20]. The most commonly used techniques are ion-exchange and gel filtration chromatography which are usually combined.

The development of techniques and methods for the separation and purification of biological macromolecules such as proteins has been an important prerequisite for many of the advances in bioscience and technology over the last decades. The development of new generations of chromatographic media with increased efficiency and selectivity as well as of new electrophoretic techniques for fast analysis of protein composition and purity have contributed the increase of knowledge about biological molecules. The application of the traditional and recently developed techniques for the isolation and separation of α- and β-amylases is decribed in some detail in this review. Among the enormous number of techniques reported in the literature for the isolation and purification of amylases through the use of classical methods, a few examples will be mentioned herein.

Glycogen precipitation is a standard method for the purification of amylases from different sources [21]. Kruger and Tkachuk [22] isolated and separated four α-amylases from hard red spring wheat using acetone fractionation, glycogen precipitation and ion-exchange chromatography. α-Amylase from the digestive juice of larvae of Indian tasar silkworm (*Antheraea mylitta*) has been purified 142-fold with a total recovery of 30% using ammonium sulphate fractionation, ethanol precipitation, glycogen complex precipitation and gel filtration chromatography on Sephadex G-100 [5]. Amylase–glycogen precipitation followed by autodigestion of the glycogen accounted for the highest purification (11.5-fold). Final purification of the amylase (2.7-fold) was

carried out by size exclusion chromatography on Sephadex G-100. The purified enzyme exhibited a single protein band on SDS–PAGE and it was resolved into five isozymes by IEF with pIs in the range 4.7–6.5. This amylase is most active at pH 9.5 and is a Ca^{2+}-dependent endoenzyme which hydrolyzes starch into maltose, maltotriose and maltotetrose.

β-Amylase from *Sorghum bicolor* has been purified by ammonium sulphate fractionation and gel filtration chromatography on Sephadex G-200, with a yield of 54% and a 5-fold increase in specific activity [23]. Roy and Hedge have reported a quick and simple chromatographic procedure for the purification of sweet potato (*Ipomea batata*) β-amylase [24]. After ammonium sulphate fractionation of the crude extract, dissolution and dialysis of the fraction precipitated in the 40–60% saturation range, ion-exchange chromatography was performed on DEAE-Sephadex A-50 in 25 m*M* phosphate buffer (pH 8.0) containing 2 m*M* 2-mercaptoethanol. Elution was performed by a NaCl gradient up to 0.5 *M*. To further improve the degree of purification achieved, the samples were rechromatographed on the same regenerated column. The enzyme recovery was 50.3% and the enrichment factor 47.8. This procedure allowed the preparation of electrophoretically pure β-amylase, devoid of contaminating activities such as α-amylase, α-glucosidase, amyloglucosidase and phosphatase which are frequently present in commercial preparations of the enzyme. In addition, the procedure can be completed in a short period. β-Amylase from the tap root of alfalfa has been purified and simultaneously separated from α-amylase activity [7]. After ammonium sulphate and ethanol fractionation of the proteins from the crude extracts, gel filtration and ionic exchange chromatography were performed. The purified β-amylase migrated as a single polypeptide on SDS–PAGE, with a MW corresponding to $57.6 \cdot 10^3$.

2. Methods for the determination of amylase activity

More than two hundred methods have been described for the determination of amylase activity. The most common of these can be grouped into amyloclastic, saccharogenic and chromogenic methods [25,26].

The amyloclastic methods measure the breakdown of a starch substrate by viscosimetric, turbidimetric, iodometric [27–29] or nephelometric procedures [30]. For a brief description of them, refer to Foo and Rosalki [25].

The saccharogenic methods measure the sugars produced in the reaction, which are determined by their reducing ability or by specific enzymatic methods. This group includes the widely used method of dinitrosalicylic acid reagent [31] or a modified version [32], and the Somogyi method [28]. An interesting variant of these methods is the so-called end-group analysis of the products which allows the determination of "true specific activities", since it measures the number of hydrolyzed bonds [33]. Since α-amylase hydrolyzes starch in a random fashion producing a complex mixture of maltodextrines, its true activity is difficult to measure quantitatively. In the end group analysis method, reaction products formed at 6-min time intervals are reduced with sodium borohydride and subsequently hydrolyzed with acid. The stoichiometric amount of sorbitol obtained corresponding to the number of glycosidic bonds hydrolyzed is determined by HPLC.

The chromogenic methods use specially synthesized substrates which yield a coloured product that can be measured spectrophotometrically. Two types of chromogenic substrates have met with considerable popularity, the so-called "insoluble" substrates and *p*-nitrophenol derivatives of oligosaccharides. Amylolytic insoluble substrates are dyes covalently bound to starch, amylose or amylopectin. The substrate-dye complex liberates soluble dye fragments as a result of amylase action, the complexes themselves being insoluble throughout or rendered insoluble by the addition of a precipitating agent. Commercially available amylase insoluble substrates include: Phadebas Amylase Test (Pharmacia Diagnostics, Uppsala, Sweden) using a highly cross-linked starch polymer dyed with Cibachrom-Blue, Amylochrome (Hoffman-La Roche, Nutley, NJ, USA), and Remazol Brilliant Blue R labelled-amylose and amylopectin (Calbiochem, San Diego, CA, USA) [34]. In the late seventies, defined substrates using *p*-nitrophenyl (PNP) derivatized oligosaccharides

were introduced into clinical analysis practice and are now increasingly popular. They have been adopted for use in microtiter plates for the specific determination of α- and β-amylase [35]. Several commercially available reagents use substrates chemically bonded to PNP through the reducing glucosyl group. Hydrolysis by the amylase, in conjunction with an α-glucosidase acting on short maltosaccharides, leads to PNP liberation, which is followed by measurement of absorbance at 405 nm. The α-amylase assay substrate is PNP-maltopentaoside blocked at its nonreducing end. β-Amylase assay substrate consists of a mixture of PNP-maltopentaoside and PNP-maltohexaoside. These substrates were used in the specific determination of α- and β-amylase in cereals [36,37]. The Pantrak reagent (Calbiochem Behring, La Jolla, CA, USA) has also been applied in the quantitative determination of α-amylase in human serum and urine; it contains PNP-pentaoside, PNP-hexaoside and about 800 units/l of microbial α-glucosidase [38]. Another commercially available kit for β-amylase determination comes from Testomar (Behring Diagnostics, La Jolla, CA, USA) [39]. The Enzyline PNP-Unitaire Kit for amylase comes from BioMérieux (Marcy l'Etoile, France). As an alternative to the chromogenic substrates, substrates which undergo a fluorogenic change when acted upon by an enzyme are useful in continuous assays. *p*-Nitrophenyl O-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-O-α-D-glucopyranosyl-(1-4)-O-α-D-glucopyranosyl-(1-4)-O-α-D-glucopyranosyl-(1-4)-α-glucopyranoside (FG5P) is an intramolecularly quenched fluorescent substrate [40]. It is resistant to α-glucosidase and glucoamylase because of its 2-pyridylamino group. Hydrolysis of its glycosidic bond uncovers latent fluorescence by the separation of the fluorogenic 2-pyridylamino group from the quenching PNP group.

Originally developed to assay the potency of antibiotics, the diffusion test on agar plates has also been applied to the determination the activities of various enzymes, such as amylases, proteases and lipases. The gel for the enzyme plate test incorporates the appropriate enzyme substrate. The properties of soluble starch and β-limit dextrin, as two alternative enzyme substrates in this test have been discussed [41,42]. The plate test for differentiation of α- and β-amylase activity has been used to identify both activities present in a malted barley [43].

In order to differentiate between acute pancreatitis and acute intraabdominal problems requiring surgery, two methods have been developed for the identification of pancreatic and salivary amylase isozymes [17,44]. These were an inhibitor assay and the use of PNP-6^5-O-β-D-galactopyranosyl-α-maltopentaoside as a substrate. The inhibitor assay (Pharmacia) utilized a wheat protein which selectively inhibited salivary isoamylase. The isozymes were differentiated by incubation of the sample with and without the wheat protein and are assayed for amylase activity using a standard chromogenic technique.

Tissue print-immunoblots on nitrocellulose membranes reveal the localization of proteins with great anatomical detail. This technique has been used to examine the anatomical distribution of sweet potato α-amylase, β-amylase, and starch phosphorylase in the root [45–47].

3. Chromatographic methods for α- and β-amylases

3.1. Salt promoted adsorption methods

The term salt promoted adsorption denotes those methods based on interactions that are reinforced by salts [48]. Almost fifty years ago, Tiselius [49] was the first to use the term "salting out chromatography". He separated serum proteins and ink dyes by paper chromatography in the presence of high concentrations of sulphates or phosphates. Today, several well studied separation methods are known to be based on interactions that are promoted by water structuring (antichaotropic) salts. The use of high salt concentration is also advantageous because it helps to prevent bacterial growth and to stabilize the tertiary structure of proteins [50]. Two of these methods will be described briefly in this Section: hydrophobic interaction chromatography (HIC) and thiophilic interaction chromatography (TIC). Immobilized metal-ion affinity chromatography (IMAC) could be included in this group, but it will be treated separately because the adsorption of

proteins to IMAC gels does not necessarily require a high salt concentration [51].

3.1.1. Hydrophobic interaction chromatography

Hydrophobic interactions are based on the tendency of non-polar molecules (or polar molecules containing large enough non polar regions) to self-associate in an aqueous medium [52]. The association is not primarily due to interactions between the non-polar solutes, but rather to the lack of affinity between water and non-polar or hydrophobic molecules. The formation of self-associated complexes is governed by the increase in entropy produced by the liberation of water formerly structured at the surface of the hydrophobic molecules involved [52,53]. Hydrophobic interactions play an important role in biological systems. They are a major driving force behind the folding of globular proteins, association of protein subunits, binding of small molecules to proteins as in enzyme catalysis, regulation and transport across surfaces. They are also responsible for the self-association of phospholipids and other lipids to form the biological membrane bilayer and the binding of integral membrane proteins [53]. The hydrophobic effect has been a matter of interest in chemistry for more than a century but it was not until 1972 that chromatography on amphiphilic beds became widely used [52]. Amphiphilic beds are those containing both hydrophobic and hydrophilic groups. The first gels of practical use for HIC were of a mixed hydrophobic-ionic character [54]. The mechanism of adsorption to mixed character gels is very complex, and accordingly the recoveries are usually low. To avoid the influence of charges, several types of neutral alkylated and arylated agarose derivatives have been synthesized [55–58]. Other new methods for immobilization of hydrophobic ligands have been developed, e.g. through attachment of alkyl sulphides to oxirane activated agarose [59]. Adsorption to these neutral amphiphilic beds is mainly due to hydrophobic interactions between the hydrophobic ligand and apolar regions on the surface of proteins (Fig. 1). The strength of the interaction depends both on the relative hydrophobicity of ligands and proteins and on the characteristics of the environment, e.g. presence of salts and polarity of the solvent [52,53]. Hydrophobic adsorption is positively correlated with ionic strength

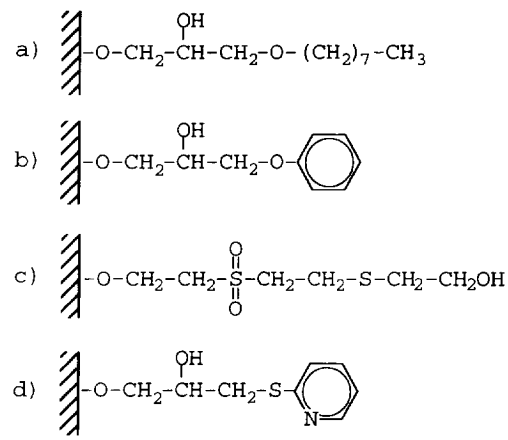

Fig. 1. Hydrophobic and thiophilic ligands coupled to a gel matrix. (a) octyl-Sepharose; (b) phenyl-Sepharose; (c) T-gel; (d) PyS-gel.

and temperature and inversely correlated with the polarity of the molecules involved. The Hofmeister series for protein precipitation is also valid for adsorption caused by hydrophobic interaction. According to their adsorption promoting effect, water-structuring salts can be arranged as follows, in order of decreasing efficiency [52]:

$$Na_2SO_4 > NaCl > (NH_4)_2SO_4 > NH_4Cl > NaBr > NaSCN$$

Desorption can be accomplished by decreasing salt concentration, changing the pH or temperature, or decreasing the polarity of the solvent with ethylene glycol or some other non denaturing alcohol. The adsorbents can be re-used several times. After each chromatographic run the adsorbents are washed with 6 *M* urea or guanidine hydrochloride to remove strongly adsorbed proteins. After washing, the gel can be equilibrated with starting buffer and used immediately for the next run. If detergents have been used, a regeneration procedure involving washing with different alcohols has to be applied. Gels can be stored at 4°C in the presence of 20% ethanol.

The relative hydrophobicities of amylases of different origins (sweet potato, soybean, malt) have been studied by comparing the chromatographic behavior of the enzymes on phenyl-Sepharose [60]. The enzymes were applied in the presence of 3 *M* sodium chloride and eluted by decreasing the concentration of sodium chloride, followed by water and

Table 3
Purification of sweet potato β-amylase on phenyl-Sepharose CL 4B

Fraction	Total proteins (mg)	Total activity (EU)	Specific activity (EU/mg)	Purification fold	Recovery (%)
Non-pigmented extract	485.0	301	0.62	—	—
Eluate from phenyl-Sepharose	7.6	237	31.18	50.3	79

30% ethyleneglycol. Soybean β-amylase turned out to be the least hydrophobic, passing unretarded through the columns of phenyl-Sepharose in the presence of 3 *M* NaCl. Sweet potato β-amylase was quantitatively adsorbed and eluted by 1 *M* NaCl. Based on this result, a protocol for the purification of sweet potato β-amylase was designed obtaining 79% recovery and a purification factor of 50-fold (Table 3). Amylases from malt were resolved into two active fractions with markedly different hydrophobicities: α-amylase was eluted by eliminating NaCl from the adsorption buffer and β-amylase was desorbed by 30% ethylene glycol.

α-Amylases from germinated maize, oats, rice and sorghum -seeds were isolated by glycogen precipitation and hydrophobic interaction chromatography on octyl-Sepharose [61]. Similar elution profiles were found for all cereal α-amylases: they were eluted by using a decreasing gradient of $(NH_4)_2SO_4$ and simultaneously increasing the gradient of ethylene glycol. The purification was mainly achieved by glycogen precipitation (43-fold), the second step allowing a further 2-fold enrichment (Table 4). A promising degree of purification was obtained for the enzymes of the four cereals with this two-step method, which is simpler than previously described procedures for rice and sorghum α-amylases. β-Amylase from tubers of ichoimo (*Dioscorea batatas*), a very popular food in Japan, was purified by a several step procedure including a final hydrophobic interaction chromatography on butyl-Toyopearl 650S [62]. After adsorption in 0.1 *M* acetate buffer, pH 6 in the presence of 45% saturation ammonium sulphate, elution was performed in 0.1 *M* acetate buffer, pH 6 by stepwise decrease in salt concentration to 10%. The purified enzyme showed a single protein band on SDS–PAGE, and two bands with amylase activity on native PAGE. About 770-fold purification was achieved over the crude extracts with a yield of only 3.4%. This low yield is mainly due to a treatment at pH 4.5 performed to precipitate most of the mucilage from the viscous crude extract.

The separation of α-amylase and glucoamylase from the culture broth of the mold *Chalara paradoxa* has been reported [63]. The procedure involved several steps: precipitation with polyethylene glycol, DEAE-cellulose adsorption and α-cyclodextrin (α-CD)-Sepharose 6B affinity chromatography (Table 5). After these steps, hydrophobic interaction chromatography on butyl-Toyopearl 650M was performed to separate α-amylase and glucoamylase; α-amylase eluted in the breakthrough fractions at 2 *M* NaCl whereas glucoamylase was retained on the adsorbent and eluted by salt elimination.

An interesting feature of HIC is that it is possible to achieve further purification even when it is performed after the affinity chromatography step.

Table 4
Purification of α-amylase from 10 g germinating maize seeds[a]

Fraction	Total proteins (mg)	Total activity (EU)	Specific activity (EU/mg)	Purification fold	Recovery (%)
Crude extract	111.5	160	1.43	—	100
Glycogen precipitate	2.06	126	61.00	43	79
Octyl-Sepharose	0.54	59	110.00	77	37

[a] Data from Ref. [61].

Table 5
Purification of amylase fractions from *Chalara paradoxa* culture filtrate[a]

Purification step	Total proteins (mg)	Total activity (EU)	Specific activity (EU/mg)	Recovery (%)
Crude enzyme	2280	22.600	9.9	100
PEG precipitate	1170	17.600	15.1	77.8
DE-52 cellulose	485	11.400	23.1	50.3
α-CD-Sepharose 6B	96	7.490	78.0	33.1
α-Amylase[b]	7	450	64.3	2.0
Glucoamylase[b]	11	620	58.5	2.7

[a] Data from Ref. [63].
[b] Butil-Toyopearl 650M fractions.

This is because HIC is based on differences in the exposed hydrophobic groups of the proteins. In addition, since there is a wide variety of HIC gels commercially available, it is easy to select the most suitable adsorbent.

3.1.2. Thiophilic interaction chromatography

In 1985 Porath et al. introduced a new type of group-specific adsorbents for protein chromatography, named thiophilic adsorbents [64]. The first gel of this kind described was the so-called T-gel which contains both a sulfone and a thioether group (Fig. 1c). The T-gel is synthesized by coupling 2-mercaptoethanol to agarose activated with divinyl sulfone. Later, Porath and Oscarsson [65], by coupling 2-thiopyridine to epoxy-activated agarose developed a new thiophilic adsorbent (PyS-gel, Fig. 1d).

When serum proteins are applied, thiophilic adsorbents show a characteristic pattern of adsorption in which mostly immunoglobulins are bound and albumin passes through. In contrast to this, hydrophobic beds bind albumin. Thiophilic gels (T-gels) have proved to be useful tools for the selective purification of immunoglobulins from various sources such as mammalian sera and ascites fluid [66]. It has also been successfully applied to the purification of monoclonal antibodies [67]. Although the mechanism of thiophilic adsorption is unknown, it seems to rely on specific interactions between electron-donor groups on the ligand, and appropriate acceptor sites on the protein surface. Like hydrophobic interaction chromatography, thiophilic interaction chromatography is a salt promoted adsorption process: proteins are adsorbed to thiophilic ligands at high concentrations of antichaotropic salts and are eluted at low salt concentration. As for hydrophobic interaction chromatography, potassium and sodium sulphates significantly increase adsorption. However, chloride anions in high concentration promote protein binding to hydrophobic gels, but markedly decrease the capacity of thiophilic adsorbents [64,66,68]. The affinity for thiophilic ligands is not restricted to immunoglobulins; other proteins such as α1-macroglobulin, papain, trypsin, and lysozyme can also be considered thiophilic [68]. The affinity of sweet potato β-amylase for thiophilic ligands was demonstrated [69]. When pure β-amylase was applied in 0.1 *M* sodium phosphate (pH 7.4) on either T-gel or PyS-gel, enzyme adsorption was low (about 10%). However, enzyme adsorption to both types of thiophilic gels was promoted by antichaotropic salts (Fig. 2). For the PyS-gel, sulphates exhibited the following series of decreasing efficiency:

$$Na_2SO_4 > (NH_4)_2SO_4 > K_2SO_4 > MgSO_4$$

Lower salt requirements and higher protein recoveries were obtained with the PyS gel. This justified its selection for β-amylase purification starting with non-pigmented extracts from sweet potatoes. The chromatographic profile obtained in the presence of 0.3 *M* sodium sulphate is shown in (Fig. 3). The enzyme was eluted as a sharp peak after salt elimination from the sample buffer, with a quantitative recovery in a very concentrated form. This one-step purification procedure for sweet potato β-amylase by TIC was highly reproducible and allowed an enrichment factor of 29 with a yield of

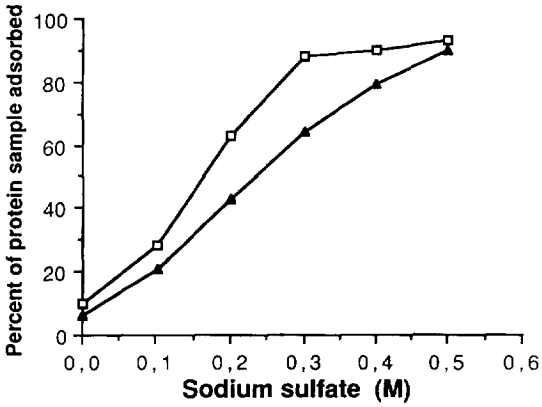

Fig. 2. Sodium sulfate effects on the thiophilic adsorption of β-amylase on PyS-gel (□) and T-gel (▲).

Fig. 4. PAGE (7.5% acrylamide, pH 8.3) at various stages in the purification of β-amylase, stained with Coomassie Brilliant Blue. Lane 1: crude extract; lane 2 unpigmented extract; lanes 3 and 4: peaks 1 and 2 from the thiophilic interaction chromatography, respectively (see Fig. 3).

56.3%. Electrophoretic analysis (Fig. 4) demonstrated the presence of a single band corresponding to the enzyme.

Amylases in malted barley extracts were fractionated by combining two salt-promoted adsorption processes: thiophilic interaction chromatography (TIC) and hydrophobic interaction chromatography (HIC) [70]. This strategy allowed the separation of two α-amylases and one β-amylase. The malt extract was first applied to the T-gel in the presence of 0.5 *M* sodium sulfate and in a separate experiment, in the presence of 3 *M* sodium chloride. There was no adsorption of amylases in the sample containing sodium chloride. Amylases were completely adsorbed when applied in the presence of sodium sulfate and were eluted by salt elimination (peak I)

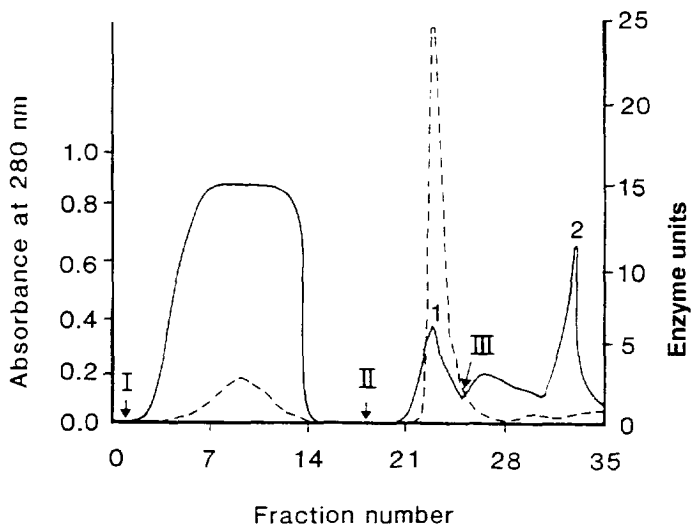

Fig. 3. Purification of sweet potato β-amylase by TIC on PyS-gel. I: Sample application; II: desorption by sodium sulfate deletion; III: desorption with 30% ethyleneglycol. Solid line, protein (A_{280}, left axis); dashed line, activity (enzyme units, right axis).

and water (peak II). Both peaks exhibited amylase activity. Pooled fractions from peak I were applied to phenyl-Sepharose in the presence of 3 *M* sodium chloride. After eliminating the salt, a fraction containing the enzyme referred to as α-amylase I was purified with an enrichment factor of 32. Elution with 30% ethylene glycol from the phenyl-Sepharose gel gave rise to a β-amylase containing fraction. The other amylasic component eluted from the thiophilic gel by water was characterized as α-amylase II. When this fraction containing α-amylase II was applied to phenyl-Sepharose in 3 *M* sodium chloride, the adsorption was so strong that the elution peak obtained by salt elimination was broad and extended. It is clear from these results that the adsorption to both thiophilic and hydrophobic gels is salt promoted, but the nature of the interactions involved are differentiated by different elution behaviors and disparate salt effects. Sodium chloride was ineffective at promoting thiophilic adsorption but it was effective for increasing protein interactions with phenyl-Sepharose. α-Amylase I and β-amylase I were co-eluted from T-gel by eliminating the salt from the adsorption buffer, and afterwards separated on the phenyl-Sepharose gel.

In conclusion, these results strongly suggest that these two salt-promoted interactions are based on different adsorption mechanisms and may thus be used sequentially.

3.2. Phenylboronate-based adsorbents

The interaction between borates and boronates with *cis*-diols has been used for the separation of biomolecules by ion-exchange chromatography and electrophoresis since the 1950s [71–73]. Upon forming the complex, borate adds negative charge to an otherwise neutral diol and this effect was exploited to achieve separations [74]. However, it was not until the early 1970s that boronate moieties were introduced as ligands for affinity chromatography. By coupling boric acid to a benzene ring, it is possible to obtain a stable boron-carbon bond. Several boronates have been tried, but *m*-amino-phenyl-boronate still continues to be the most used ligand (Fig. 5); see Ref. [75] for a review. The ideal pH for complexation depends on the pK_a of the ligand which is usually above 8.5, and this restrics the

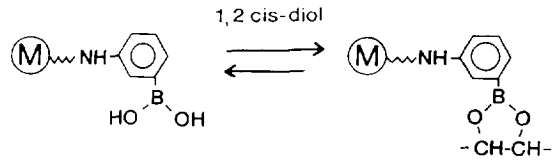

Fig. 5. Interaction of phenyl-boronate ligand with 1,2-*cis* diols.

applications of the technique. The nature of the matrix, the coupling chemistry and the ligand concentration have a profound effect on the chromatographic properties of boronate-beds. Immobilized phenyl-boronates have had numerous applications, including the separation of nucleotides and oligonucleotides from their deoxy-forms, catechol estrogens from other hormones, different sugars, and the determination of non-enzymatically glycosylated proteins to monitor diabetic patients [75]. Because of the benzene moiety of the phenylboronates, the separation of glycoproteins, polynucleotides, and other macromolecules present serious problems due to non-specific binding via hydrophobic and π-π interactions [74,76]. Interestingly, because of these so-called secondary interactions, phenyl-boronate ligand can be used successfully for the purification of non-glycosylated proteins. This was the case with soybean β-amylase, a non-glycosylated protein, which bound so strongly and reversibly to the adsorbent that it was possible both to immobilize it and to purify it from a crude extract. For the purification by phenyl-boronate chromatography the sample was applied in 0.1 *M* sodium phosphate (pH 6.8), and eluted through a gradient of a competing *cis* configuration poliol (sorbitol) [77,78]. The purification factor obtained in this step was 8.2 with a recovery of 72%. Analogous to other salt promoted methods, the adsorption capacity of the adsorbent was increased substantially by antichaotropic salts.

3.3. Immobilized metal-ion affinity chromatography

Immobilized metal-ion affinity chromatography is a separation technique based on a specific and reversible interaction between an immobilized metal-ion and the protein [79]. The method was introduced by Porath et al. in 1975 [80] and since then, copper, nickel, zinc, cobalt, iron, and calcium immobilized on agarose derivatized with several chelating ligands

have been used successfully for the purification of proteins from complex extracts and biofluids [81–85]. Peptide adsorption studies by Belew and Porath revealed histidine, cystein and tryptophan to be the most important amino acids involved in the coordination bond with the immobilized metal-ion [86]. Proteins are eluted either by changing pH or by including competitive agents such as imidazol. The separation of wheat amylases by IMAC has been reported [87]. A crude extract from germinated wheat was applied to a column with Cu-iminodiacetate-Sepharose. Separation of high from low isoelectric point wheat α-amylases was obtained by changing the concentration of glycine in the elution buffer. High-p*I* α-amylases (about 84% of wheat amylases) were mainly eluted with 100 m*M* glycine whereas low-p*I* amylases were eluted earlier. Although IEF patterns of the main amylase fraction eluted with 100 m*M* glycine revealed some contaminant proteins, a purification factor of 20 was obtained and the preparation was free from β-amylases and low p*I*-amylases. Despite its versatility, the use of IMAC for the purification of amylases is not widespread.

3.4. Biospecific affinity chromatography

Affinity chromatography relies on the ability of proteins to bind specifically and reversibly to other compounds. Examples of biospecific phenomena include the interactions between enzyme and substrate or inhibitor, hormone and receptor, antigen and antibody, and between glycoprotein and lectin [88]. One of these molecules, termed the ligand, is immobilized on the solid-phase while the other, the counterligand, is adsorbed when the extract is applied to the chromatographic column [89,90]. Affinity chromatography is usually a very powerful separation method, especially when the protein to be purified is a minor component of the starting material. Interestingly, the first application of affinity chromatography was the selective adsorption of amylase on to insoluble starch in 1910. In this review, the term affinity chromatography will be used strictly to include those methods based on biologically functional pairs. In this context, a group of very significant protein-protein interactions is the antigen-antibody pair which gives rise to a technique called immunosorption.

3.4.1. Use of competitive inhibitors (cyclodextrins)

Cyclodextrins are cyclic oligosaccharides enzymatically derived from starch. Three main cyclodextrin types have been identified: α-, β-, and γ, composed of six, seven and eight glucopyranose units respectively, linked by α-1-4 glycosidic bonds. Due to their cyclic nature, their outer surface is hydrophilic and the inner cavity is non-polar. The internal cavity can retain guest molecules of the appropriate dimensions forming an inclusion complex in which the encapsulated molecule is protected. This is the basis of their widespread use in the pharmaceutical and food industries. Cyclodextrins are also used to stabilize certain proteins and to reduce losses of enzymatic activity due to heating, freeze drying, aggregation, storage or oxidizing agents [91]. Cyclohexa- and cycloheptaamylose act as competitive inhibitors of α- and β-amylases [92,93]. In view of the high affinity of these enzymes for cyclohexaamylose (α-CD)- and cycloheptaamylose (β-CD), the cyclodextrins started to be used as ligands for the purification of amylases. Vretblad et al. demonstrated that (α-CD)-Sepharose 6B adsorbed crystalline sweet potato β-amylase [94]. Later, the binding of amylases (from pancreas, sweet potato, *Aspergillus*) and amyloglucosidase to immobilized α-CD was studied [95].

A two-step purification procedure including ion-exchange chromatography on DEAE-Sephadex and chromatography on α-CD-Sepharose was developed for sweet potato β-amylase starting from a crude extract [96]. The enzyme produced after the affinity chromatography step was homogeneous according to PAGE.

Immobilized cyclodextrin affinity adsorbents have been used to study the amylolytic enzymes in plant tissues. The amylases usually exist in multiple forms but the physiological role of this multiplicity has not been established.

Silvanovich and Hill in 1976 developed a procedure for the purification of α-amylase from a wheat-rye hybrid, Triticale [97]. The method consists in glycogen precipitation followed by selective adsorption to agarose substituted with β-CD. The glycogen precipitation was used mainly to produce

discoloration and a preliminary purification (26-fold). However, this preliminary treatment recovered only 36% of the activity. The subsequent affinity chromatography step gave a yield of 92% of the applied activity, with a purification of up to 180-fold over the crude extract (Table 6). Amylases in cultured rice cells were studied and compared to those of intact seeds [98]. In this study, two groups of intracellular α-amylase isozymes were purified. By native PAGE, three isozymes were detected, α-amylase I, II and III. Purification of the three amylase activities was performed, first by ion-exchange chromatography on a DEAE-Sephacel column. Secondly, α-amylase I and II were further purified by affinity chromatography on a α-CD-Sepharose 6B column. Both amylases gave a single protein band on subsequent native PAGE and SDS–PAGE. However, activity staining of amylase II on the polyacrylamide gel revealed two bands. Amylase III was obtained as a homogeneous protein as analyzed by native PAGE and SDS–PAGE after gel permeation on Toyopearl HW-60F and affinity chromatography on β-CD-Sepharose 6B. The final purification factors for amylases I, II, and III, after the affinity chromatography steps were 6.4, 2.6, and 10.7 respectively: Cycloheptaamylose (β-CD)-Sepharose 6B has specific affinity for amylase-III. Amylases I and II were adsorbed by (α-CD)-Sepharose 6B but not by (β-CD)-Sepharose 6B. Action patterns on soluble starch indicate that these enzymes are all α-amylases. Other amylase systems from plant tissues contain both α- and β-amylases [99]. In cultured rice cells and other rapidly growing tissues it appears that only α-amylases are required.

Totsuka and Fukazawa [100] have reported a method for the affinity purification of soybean and barley β-amylases on immobilized α-CD-Sepharose 6B, in the presence of 1–2 *M* ammonium sulfate.

Table 7
Effect of ammonium sulfate concentration on the adsorption of various β-amylases to α-CD-Sepharose 6B[a]

$[(NH_4)_2SO_4]$	Soybean	Barley	Sweet potato
0.0	1	3	122
0.5	17	18	138
1.0	119	114	144
2.0	158	152	151

[a]Data from Ref. [100].
Data are given in Enzyme Units per ml of packed α-CD-Sepharose 6B.

The adsorbed enzyme can be eluted by salt elimination. This is advantageous since it is not necessary to include the counter ligand α-CD, which is expensive and must be removed afterwards, in the buffer. By this procedure, soybean β-amylase was purified 10-fold to homogeneity. The authors have demonstrated that only ammonium sulphate (1–2 *M*) assists specific binding (Table 7); other salts such as 0.5 *M* sodium sulfate, 1 *M* sodium chloride or 1 *M* sodium nitrate do not.

In order to determine whether the major endo-amylases found in pea leaves, stems, and cotyledons, were identical or isozymic forms, the most abundant α-amylase in shoots and cotyledons from pea (*Pisum sativum L.*) seedlings were purified [101]. The purification factors were 6700 and 850 respectively, utilizing affinity chromatography (cyclodextrins), gel permeation and ultrafiltration. The purified amylases appeared to be identical as judged by the characteristics studied: MW, p*I*, pH activity profile, kinetic properties, substrate specificities and one-dimensional peptide fingerprints. α-Amylase from germinating seedlings of alfalfa was partially purified by biospecific affinity chromatography on β-CD Sepharose 6B [102]. The extract was adsorbed on to an affinity gel at 4°C. α-Amylase adsorbed to the column was

Table 6
Purification of α-amylase from malted triticale by glycogen precipitation and β-CD-Sepharose 6B affinity chromatography[a]

Fraction	Total protein (mg)	Total activity ($EU \times 10^{-6}$)	Specific activity (EU/mg)	Purification fold	Recovery (%)
Crude extract	2904.0	4.67	1610	—	—
Glycogen precipitate	40.0	1.67	41 750	26	36
Affinity peak	6.6	1.52	230 000	143	33

[a] Data from Ref. [97].

eluted with a solution of β-CD. The purification factor was 831 and the yield 7.8%. Similarly, α-amylases from malted barley and from the incubation medium of barley aleurone layers were purified by affinity chromatography on β-CD Sepharose 6B [33,103]. The cyclodextrin used for elution was removed by dialysis [103]. The distribution of amylases within sweet potato (*Ipomea batata*) root tissue was studied by tissue printing techniques, and the enzymes were characterized after purification by affinity chromatography [45]. α-Amylase was purified on β-CD-Sepharose and β-amylase on α-CD-Sepharose. The purification factors were 662 and 24 for α- and β-amylases, respectively. The cyclodextrins were removed by gel permeation on Sepharose-4B.

Ostrich pancreatic α-amylase was purified to homogeneity in a single step by a cycloheptaamylose (β-CD)-Sepharose 4B affinity chromatography procedure [104]. The extract was adsorbed batchwise to the affinity gel in the presence of a protease inhibitor, and the adsorption mixture was subsequently packed in a column. After thorough washing with 50 m*M* acetate buffer (pH 5.5), 1 m*M* CaCl2, the ostrich pancreatic α-amylase was eluted by a 8 mg/ml β-CD solution. The purification of the enzyme by a single step affinity procedure was highly successful in that both a homogeneous preparation and a high yield were obtained. The enzyme was purified by a factor of 17 with a yield of 76%. No minor contaminants could be detected on SDS–PAGE. The first 53 amino acids of the N terminus of the protein were sequenced after deblocking the amino terminus with pyroglutamyl peptidase. Ostrich pancreatic α-amylase showed 72% identity with mouse, rat, porcine and human pancreatic α-amylase. The kinetic parameters (K_m, k_{cat} and k_{cat}/K_m) for the hydroysis of linear malto-oligosaccharides was determined. From these data, an active site profile was established for ostrich pancreatic α-amylase. In addition, inhibition by monosaccharides, β-CD, and wheat α-amylase inhibitor was studied.

In spite of the selectivity of cyclodextrins, they have seldom been used so far to separate amylase isozymes. It may not be easy to achieve separation of isozymes, nor of very similar amylases, by cyclodextrin affinity methods, because of their relatively broad specificity.

3.4.2. Use of starch or other substrates as chromatographic adsorbents

Polysaccharide-degrading enzymes have been isolated from various sources by adsorption to substrates [21]. Some examples are described in Section 1.3. Newer chromatographic procedures with immobilized substrates achieve better purification factors and yields in many cases. Wheat α-amylase has been purified 93-fold in a single step by adsorption to glycogen-Sepharose and elution with glycogen in solution. Recovery was 84% and the purity was about 90%, according to polyacrylamide gel electrophoresis [105]. The procedure compares favorably to the more laborious method based on glycogen precipitation reported by Kruger and Tkachuck [22]. An efficient affinity chromatography method for the purification of β-amylase from mustard cotyledons on starch columns was reported [106]. To obtain a rigid matrix, suitable for packed bed chromatography, water-soluble polysaccharides are made insoluble by a crosslinking reaction. Epichlorohydrin is the most frequently used cross-linking reagent in these reactions. Cross-linked starch or starch derivatives are useful affinity adsorbents for the isolation of bacterial α-amylases [107]. A suitable cross-linked starch was synthesized by optimizing the important parameters in the cross-linking reaction (epichlorohydrin concentration, ethanol–water ratio, NaCl–epichlorohydrin ratio, reaction time and temperature). Beaded affinity adsorbents have been developed in which the gelling properties of the alginate are combined with the adsorption properties of the cross-linked starch. Almost no enzymic breakdown occurs, while the binding capacity is approximately 12 units/mg of adsorbent [108]. To obtain beaded adsorbents based on starch and alginate, two different strategies were followed: either cross-linked starch was incorporated into an alginate matrix by Ca^{2+} complexation or a co-polymer of alginate and starch was synthesized in which alginate was the building frame for the Ca^{2+}-complexed polymer mixture and the bead was subsequently cross-linked with epichlorohydrin (Fig. 6). Both methods yield affinity adsorbents with comparable affinity characteristics. The adsorption of different commercially available α-amylases and a number of other starch-degrading enzymes has been investigated. Bacterial α-amylases and sweet potato β-amylase are readily

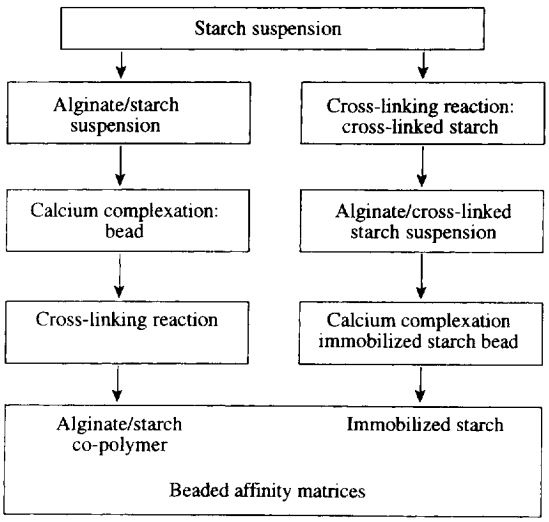

Fig. 6. Schematic representation of two procedures for the preparation of beaded affinity adsorbents for α-amylases (adapted from Ref. [107]).

bound to the adsorbents, whereas *Aspergillus oryzae* amylase, pullulanase and amyloglucosidase are not adsorbed under the conditions chosen. Heat stable α-amylases like the one from *Bacillus licheniformis* were conveniently eluted by temperature shift, while other α-amylases can be eluted by addition of a dilute limit-dextrin fraction. In this way, commercially available enzymes were further purified. Furthermore, the beaded form of these affinity adsorbents should allow direct use on non-clarified broth or homogenate (e.g. in a fluidized bed reactor).

3.4.3. Immunoaffinity techniques

Daussant and Bureau [109] discussed the advantages and limitations of immunoaffinity chromatography for enzyme analysis in barley and malt. Immunoaffinity chromatography is of course a very selective technique, but the elution step is usually performed under drastic conditions that are known to denature amylases. However, the method has been applied successfully in two different situations: (i) as a specific molecular filter to eliminate α-amylase from malt extracts in order to quantify β-amylase, and (ii) as an example of enzyme purification in one step from the whole grain extract, using barley β-amylase as a model. In this case, elution from the immunosorbent was efficiently achieved with preservation of enzymatic activity by preincubating the column with distilled water. The purification of *Bacillus licheniformis* thermostable α-amylase to apparent homogeneity by immunoaffinity chromatography, was reported by using an immobilized antibody prepared by immunizing rabbits with the enzyme purified by a multistep procedure and subsequently cutting out the bands from the polyacrylamide gel [110]. The yield was 86%, higher than that of the conventional multistep procedure. The antibody against *Bacillus licheniformis* thermostable α-amylase cross-reacted with Taka amylase. Proteins with similar functions in different species often have a high degree of homology in their amino acid sequences and therefore exhibit cross-reactivity to antibodies. Using this principle, Katch and Terashima, successfully purified rice α-amylases produced by recombinant yeast cells, in one step from the fermentation broth [111]. Antibodies raised against partially purified barley α-amylase were immobilized to CNBr-activated Sepharose 4B. Upon elution by 2.5 *M* NaSCN, pH 5.0, the recovery was 75% and the purification factor 2000. However, by elution with 50 m*M* sodium acetate pH 3.6, the elution peak was broad and the recovery was lower. Cross reactive antibodies thus proved useful for purification of recombinant proteins foreign to host cells. In addition, since impurities contained in fermentation broth are generally different from those in the antigen used for immunization, it is not necessary to use highly purified antigens.

3.5. High-performance liquid chromatography

In traditional low-pressure chromatography, beads with a diameter of approximately 100 micrometer are usually standard. The adaptation of HIC, IMAC as well as other protein purification techniques to the high-performance mode has been made possible by the production of beads of small and uniform size. The smaller the bead size the better and faster the separations. In this approach, higher pressure drops are required and so, only rigid particles can be used. High-performance packings have been based on silica, organic polymers, and heavily cross-linked agarose [53]. Human salivary α-amylase isozymes were rapidly separated from each other by high-

performance chromatography [40]. Whole human saliva was applied to a TSK-G 3000-SW (ToyoSoda) prepacked column. The α-amylase activity was resolved into two peaks of molecular mass 62 and $59 \cdot 10^3$, respectively. Afterwards, HPLC-chromatofocusing of each peak was performed on a Mono P prepacked HR 5/20 column (Pharmacia) and six isozymes (three major and three minor) were separated. The recovery of α-amylase activity from each HPLC column was 97–98%.

Two forms of β-amylase from taro tubers have been purified by a series of column chromatographies in 20 m*M* Tris buffer, pH 8.5 including ionic exchange on DEAE-Toyopearl 650 S, gel permeation on Toyopearl HW 50F and high-performance liquid chromatography on Mono Q HR 5/5, followed by, re-chromatography on this last column [8]. Both taro β-amylases were homogeneous as judged by SDS–PAGE, with MW of 66 and $67 \cdot 10^3$, respectively. Enrichment factors achieved were 28- and 22-fold respectively over the crude extracts, with a recovery of only 1% each. This low recovery of amylase activity may have been due to inactivation of the enzymes. The rapid separation of α-amylases from barley by high-performance ion-exchange chromatography on both porous and non-porous media has been reported [112]. Barley crude samples containing α-amylases in 5 m*M* Tris buffer, pH 8.6, 1 m*M* calcium chloride, were chromatographed on two different anion-exchange resins: Bio-Gel TSK DEAE-5-PW column (Toyo Soda) and non-porous MA7P cartridge (Bio-Rad). Two main peaks of α-amylase were separated, and there were several minor components. In the case of the porous support the analysis of a sample and regeneration of the column for the next run required about 1 h, and activity recovery was 82%. The non-porous support allowed total analysis times of less than 10 min with flow-rates of 2 ml/min, and less than 5 min at 5 ml/min. The separation was improved by using a shallow gradient. Recoveries of α-amylase activity were higher (90%) with the non-porous support. Most proteins did not bind to these columns and were washed out before the gradient started; a 10-fold purification of α-amylase activity was achieved. The α-amylase inhibitor from barley is also an inhibitor of wheat α-amylase. This interaction was studied by comparing the HPLC anion-exchange chromatograms obtained on MA7P for wheat α-amylase and for the complex enzyme-inhibitor [113].

High-performance-hydrophobic interaction chromatography (HPLC-HIC) is a relatively new addition to the rapidly advancing methods for protein separation. The HIC technique has been adapted to the HPLC mode using both the traditional gel material agarose with strong cross-linking, as well as organic polymers and silica based matrices. The retention mechanism relies essentially on the same forces as in reversed-phase chromatography. In both instances hydrophobic functional groups are bound to the support 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 may lose some of their activity. The functional groups in HIC packing materials are much more sparsely distributed, so that elution can be accomplished by buffers without appreciably denaturing the proteins.

In order to separate native α-amylases from among inactive proteins present in amylase preparations of industrial use, HPLC-HIC was performed on a propyl-SynChropak column [114]. Elution was achieved by a linear decreasing salt gradient (1.0–0.1 *M* ammonium sulphate) in 0.02 *M* potassium phosphate, pH 7.0. The chromatograms obtained were used for quality control of some industrial proteins. Additionally, HPLC-HIC proved to be an efficient method for preparative purposes producing enzyme preparations of high purity and high specific activity.

The use of HIC with mildly hydrophobic ligands such as PEG covalently attached to suitable supports for high-performance techniques is a new approach with interesting possibilities. α-Amylase has been purified from a crude preparation of porcine pancreas on a Ether-5PW column. This is a resin-based hydrophilic support containing oligo(ethylene glycol) moieties [115]. Up to 1.5 g of α-amylase crude sample could be applied to a preparative column (200×55 mm internal diameter) without loss of separation efficiency. α-Amylase of high purity was obtained, with 90% recovery. Ether-5PW proved to be very useful for the purification of pancreatic α-amylase under mild elution conditions (linear

gradient of sodium sulphate from 0.66 *M* to zero in 0.1 *M* phosphate buffer, pH 7.0) with high resolution and recovery. HIC with mildly hydrophobic ligands was used to provide qualitative information on the partitioning of proteins in PEG-salt aqueous two-phase systems [116]. It was demonstrated that the interaction of commercial α-amylase with a PEG-silica column is promoted by moderate concentrations of salts; concomitantly, the enzyme also shows strong PEG phase preference during partition. Studies of the behaviour of many model proteins showed a very good correlation in all cases between HIC and aqueous two phase partition. The application of automated analytical HIC techniques in the preliminary design of partitioning steps, could be of benefit in the development of PEG-salt aqueous two phase partitioning, a purification technique with great potential for scale-up.

3.6. Other methods

Traditionally, polymorphic forms of enzymes are separated by techniques that exploit differences in molecular charge properties such as electrophoresis, isoelectric focusing, ion-exchange chromatography and more recently chromatofocusing. In chromatofocusing, proteins are separated as a result of the isocratic formation of internal pH gradients on ion-exchange columns. This technique can discriminate between proteins which have similar net surface charge, but vary in surface charge distribution. It is thus possible to resolve by chromatofocusing proteins that cannot be separated by isoelectric focusing [117].

The appearance of α-amylase isozymes during kernel development in wheat has been studied by electrophoresis, isoelectric focusing and immunochemical techniques [118]. The development of β-amylase activity and polymorphism in wheat seedling shoot tissues was studied by electrophoresis, using polyacrylamide gels containing amylopectin [119]. Kohono and coworkers reported the purification to homogeneity of five isoforms of β-amylase from alfalfa seeds (*Medicago Sativa L.*) by chromatofocusing and cation-exchange chromatography. The five isoforms had slightly different isoelectric points (in the range 5.05 to 4.77) but their molecular mass was the same ($61 \cdot 10^3$) on SDS–PAGE [120]. β-Amylase from germinated maize grains was isolated and purified by ammonium sulphate precipitation, chromatofocusing and adsorption chromatography on hydroxyapatite [121]. After applying the sample at pH 6.2 on the chromatofocusing column, β-amylase was eluted as a single peak (average elution pH 4.15). Several α-amylase bands were detected in the starting material and the ammonium sulphate fraction, but they were absent after chromatofocusing. Minor contaminant proteins still present were then removed completely by hydroxyapatite adsorption chromatography, with elution by phosphate (β-amylase eluted in a single peak around 12 m*M* phosphate). Throughout this procedure, a 418-fold total enrichment ratio was achieved, with a yield of 26% (Table 8). The results from maize β-amylase differ widely from those reported for the well characterized endosperm β-amylase of wheat, barley, or rye. The barley enzyme, which represents nearly 2% of the total proteins of a germinating grain, is often assumed to be mainly a storage enzyme, whereas maize β-amylase constitutes a minor protein of the grain (0.2% of soluble proteins). Maize β-amylase is monomeric and its polymorphism is low.

Table 8
Purification of β-amylase from 40 g germinating maize grains[a]

Fraction	Total proteins (mg)	Total activity (EU)	Specific activity (EU/mg)	Purification fold	Recovery
Crude extract	604	397	0.66	—	100
$(NH_4)_2SO_4$ precipitate	110	320	2.91	4.4	81
Chromato-focusing	ND[b]	192	—		48
Hydroxyapatite	0.38	105	276.00	418.2	26

[a]Data from Ref. [121].
[b]Not determined, owing to Polybuffer interference.

4. Final remarks

Amylases are enzymes of great importance in the food industry. They have therefore been the subject of intense applied as well as basic reseach. Since the purification of an enzyme is a prerequisite to its subsequent characterization and to the development of industrial applications, this is an active research field. The choice of purification protocol naturally depends on the intended use, the highest purity usually being required for basic purposes in which even separation of isozymes may be important. The purity, and the yield attained, depend on the number of steps and separation techniques employed. In this respect, biospecific affinity chromatography is a key method for the preparation of homogeneous amylases, both as a single step, or in combination with other general or specific techniques. In particular, immobilized cyclodextrins probably offer the simplest option. It is noteworthy that most amylases are inhibited either by α- or β-cyclodextrins. In some cases, adsorption requires the presence of ammonium sulfate, elution is thus performed by salt elimination which constitutes a definite advantage over the specific elution by the cyclodextrins in solution. Additionally, scale-up of the affinity technique with immobilized cyclodextrins appears to be feasible.

Due to its high selectivity, immunoaffinity chromatography has been applied, on a laboratory scale, for the preparation of highly purified amylases. However, the use of protein molecules as affinity adsorbents is costly and although technically feasible, is often unrealistic on economic grounds.

The separation of polymorphic forms of enzymes is carried out by techniques that discriminate between proteins with slight differences. This is usually impossible by affinity chromatography. The method of choice would be chromatofocusing which can resolve between proteins that are not well separated by isoelectric focussing.

A fair degree of purification can be obtained from crude extracts for several plant and bacterial amylases by HIC in both low-pressure and HPLC modes. Chromatography on non-porous anion-exchange columns provides a rapid and quantitative method for the analysis of multiple forms of α-amylases. This method may also have potential for the rapid purification of α-amylases as a preparative technique. Non porous columns have several advantages, as high flow-rates are possible with good resolution and equilibration with solvents is rapid, allowing fast recycling. Good separations have been achieved with the use of the new thiophilic adsorbents. The high capacity exhibited and the possibility of reusing the bed several times after washing, make thiophilic interaction chromatography a promising technique fot the fractionation of amylases.

Recent advances in the understanding of the physical and functional properties of amylases, and of the selectivity and capacity of the adsorbents, have led to greater rationality in the design of separation methods. However, the potential of the methods for the separation of amylases has not been fully exploited. It is possible to increase the overall resolution and efficiency of chromatographic techniques by using several different adsorbents, based on different separation principles, in tandem-coupled adsorption beds [122]. Use of several beds which have different affinity characteristics, forming a multiaffinity "cascade" system, can also effectively save time, minimizing the overall number of operations, such as buffer changes, adsorption and desorption.

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