Critical Reviews in Biotechnology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ibty20

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To cite this article: Marcin Kublicki, Dominik Koszelewski, Anna Brodzka & Ryszard Ostaszewski (2022) Wheat germ lipase: isolation, purification and applications, Critical Reviews in Biotechnology, 42:2, 184-200, DOI: 10.1080/07388551.2021.1939259

To link to this article: https://doi.org/10.1080/07388551.2021.1939259

CRITICAL REVIEWS IN BIOTECHNOLOGY
2022, VOL. 42, NO. 2, 184–200
https://doi.org/10.1080/07388551.2021.1939259

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REVIEW ARTICLE

Wheat germ lipase: isolation, purification and applications

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ABSTRACT

In recent years, wheat germ lipase (WGL) is attracting considerable interest. To date, several WGL applications have already been described: (i) fats and oils modification; (ii) esterification reactions in organic media, accepting a wide range of acids and alcohols as substrates; (iii) the asymmetric resolution of various chiral racemic intermediates; (iv) more recently, the promiscuous activity of WGL has been shown in carbon-carbon bond formation. To date, no crystallographic structure of this enzyme has been published, which means its activity, catalytic potential and substrate scope is being assessed empirically. Therefore, new catalytic activities of this enzyme are constantly being discovered. Taking into account the emergency and the current interest in environmentally sustainable processes, this review aims to highlight the origin, isolation, stabilization by immobilization and applications of the wheat germ lipase.

HIGHLIGHTS

- Wheat germ as an inexpensive source of biocatalysts
- Wheat germ lipase an efficient catalyst for various chemical transformations
- Wheat germ lipase in food production
- Industrial applications of wheat germ lipase
- Wheat germ lipase as a promiscuous biocatalyst
- Immobilization of wheat germ lipase as a method of stabilization

ARTICLE HISTORY
Received 24 September 2020
Revised 10 February 2021
Accepted 7 April 2021

KEYWORDS
Wheat germ lipase; immobilization; esterification; hydrolysis; enzymatic resolution; enzyme promiscuity

Introduction

Wheat germ lipase (triacylglycerol acyl hydrolase EC 3.1.1.3) has several advantages over commonly investigated enzymes. It is obtained from largely underutilized side products of dry wheat grain milling, wheat germs. They are safe for human consumption and an abundant source of essential amino acids, antioxidants and minerals, and have found application in health foods, supplements and cosmetics. During the recent decade, wheat germ lipase has received attention due to the discovery of its ability to catalyze reactions of non-natural substrates [1]. Its yet undiscovered capabilities are certainly going to be utilized in various branches of green chemistry and biotechnology. The source plant does not need to be genetically modified to produce economically viable germ lipase, therefore limitations on GMO do not need to be considered in the enzyme's industrial applications.

Several lipases used in biotechnology are produced from organisms, which are known to cause allergies, dangerous infections or cancer - *Pseudomonas cepacia, Candida rugosa* and *Mucor miehei* [2–5]. Therefore the safe and reliable preparation of lipases derived from these organisms is more expensive, and requires microbiological expertise and certifications [6,7]. Substances produced with the use of these enzymes must be analyzed for the presence of fungal or bacterial toxins and contaminants.

Wheat (*Triticum*), first domesticated in the area of the Fertile Crescent around 9600 BCE [8], is the second most widely cultivated cereal plant after maize. It had an estimated global production of approximately 773.5 million metric tons in 2017 (including 134.3 million metric tons produced in the People's Republic of China, the largest producer), followed by rice (769.8 million metric tons) and barley (149.1 million metric tons) [9]. The annual world deposit of wheat germ is approximately 25 million metric tons [10].

The first description of hydrolytic activity in wheat grain was published by Sullivan and Howe in 1933 [11]. This was followed by Singer and Hofstee in 1948 with a method of enzyme isolation from wheat germs, and an observation that the obtained biocatalyst is capable of

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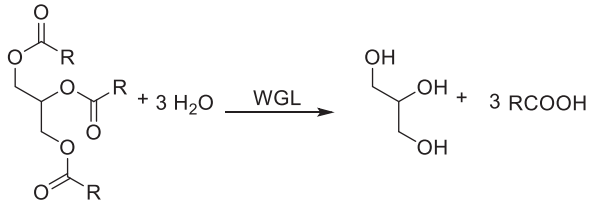


Scheme 1. WGL catalyzed hydrolysis of glycerides.

hydrolyzing both glycerides and simple carboxylic acid esters (Scheme 1) [12]. Singer's procedure has become a standard for commercial preparation of lyophilized wheat germ lipase [13]. Among others, it is available from Sigma-Aldrich and Creative Enzymes in the form of a lyophilized powder with over a 70% protein content, and an activity of 5–15 units/mg protein. In this case one unit could hydrolyze one microequivalent of fatty acid from a triglyceride in one hour, at pH 7.4 at 37 °C. Although Sigma-Aldrich uses the term 'Lipase from wheat germ, Type I' for their product, to the extent of our knowledge, no other types of this biocatalyst have been described in the literature.

Purification of wheat germ lipase

The specific activity, as well as the stability of enzymes is related to their degree of purification. Enzyme-containing extracts obtained from wheat germ contain a multitude of other components. These are: water, fats, carbohydrates, salts, proteins and other constituents of the membrane to which the enzyme is believed to be bound to [14]. Removing these components yields an enzyme, which is more homogenous and has a higher specific activity. However, certain molecules present in the native environment of the enzyme bestow stabilization on it, and in their absence the protein becomes more prone to inactivation.

Several years after the initial publications by Singer and Hofstee, Stauffer and Glass have separated crude wheat germ lipase into three fractions [15]. They demonstrated varied reactivity toward: triacetin, tributyrin and monoolein, and the oil/water interface had a different influence on their catalytic properties.

Wheat germs were, at first, extracted with hexane to remove oils and oil-soluble contents, then ground in a Wiley mill and extracted with water at room temperature. Dialysis produced an enzyme solution which demonstrated lipolytic activity of 0.15 U/mg.

The remaining supernatant was saturated with ammonium sulfate, adsorbed on calcium phosphate gel and dialyzed. The obtained enzyme demonstrated high activity toward tributyrin, up to 1.36 U/mg, which represents a nearly 38-fold degree of purification. The activity toward esters of shorter or longer length carboxylic acids was significantly lower.

Eventually, the supernatant formed above the tributyrinase-containing precipitate was further purified *via* saturation with solid ammonium sulfate. The obtained esterase demonstrated a specific activity of 0.11 U/mg and the degree of purification was 2.32. However, moving-boundary electrophoresis showed that the enzyme obtained this way is still heterogenous.

In the WGL purification procedure described by Kapranchikov et al. [16], ground wheat germ was extracted with acetone at −10 °C according to Anan'eva and Rudyuk producing an acetone powder [17]. This was extracted with a solution containing Tris-HCl, beta-mercaptoethanol and EDTA, subjected to isoelectric precipitation, gel filtration, and eventually DEAE-cellulose and gel chromatographies. The protein fraction thus obtained produced only a single peak in PAGE and in Sephadex G-150 gel chromatography. The molecular weight of the enzyme was estimated at 143 ± 2 kDa. Additionally, this peak concised with all the protein yield and enzymatic activity retained at this point, therefore it was declared nearly homogenous.

The activity of the obtained lipase was measured by titration of oleic acid, released from emulsified olive oil. The eventually achieved degree of purification was reported to be over 61-fold. The purified enzyme exhibited a specific activity of 622.5 nmol min^{-1} mg^{-1}, and the optimal conditions for its activity were 37 °C and pH 8.0.

Another purification method was described by Köse et al., employing cryogel affinity chromatography [18]. Wheat germ acetone powder was extracted with PBS, saturated using $(NH_4)_2SO_4$ and adsorbed on the cryogel. The changes in the specific activity in the course of purification were measured by the Bradford method and with a spectrophotometric assay using *p*-nitrophenyl palmitate. It was determined that purification with the use of an unmodified gel led to a 2.74-fold increase in specific activity, while the use of a Cu(II)-modified cryogel afforded a 2.97-fold increase.

It is evident from the described experiments, that the specific activity and the activity profile of the obtained enzyme depends on the method of purification. It is, however, difficult to fairly compare these methods due to the variety of employed assay methods and substrates. Unfortunately, despite the existence of effective methods of protein purification, such as nanoparticle embedded cryogels reported by Erol et al., they were not used to purify wheat germ lipase, what could positively affect its activity [19–23].

The amino acid sequence of WGL

Wheat germ lipase belongs to α/β serine hydrolases – enzymes characterized by a common sequence of α-helices and β-strands as it was described by Ollis et al. (Figure 1) [24]:

Two amino acids sequences of WGL derived from two cultivars of wheat have been determined by Saunders et al. and submitted to the UniProtKB/TrEMBL database under the accession numbers Q8L5T0 and Q8L6B0 [25]. The first one is presented below and corresponds to a theoretical molecular weight of 39,258 Da (Figure 2).

Based on the sequence and topology similarities between the hydrolases recorded in the ESTHER database, the WGL catalytic center has been assigned to the triad of amino acids Ser174, Asp230 and His293 [26]. To the extent of our knowledge, the crystal structure of wheat germ lipase has not been yet determined. However the SWISS-MODEL allows for three-dimensional visualization of the central part of the sequence (denoted in red above), including the putative catalytic triad (denoted in bold font) (Figure 3) [27–32].

Stability of wheat germ lipase

During storage, germination of wheat grain leads to hydrolysis of naturally occurring glycerides into glycerol and fatty acids by the action of wheat germ lipase. This results in loss of nutritional value and undesirable processing properties and needs to be avoided for purposes of food production. This can be prevented by thermal denaturation of lipase, however the conditions for this need to be carefully optimized in order to minimize side reactions, such as the degradation of antioxidants [33,34].

Stauffer and Glass have studied the thermal stability of the obtained esterase and tributyrinase fractions of WGL. Heating them in water solutions at 44 °C – 60 °C demonstrated, that the inactivation reactions do not follow the same kinetics order. At pH 7.2, the inactivation of the esterase fraction is a second-order reaction, while that of tributyrinase is a first-order reaction. The respective heats of activation of these reactions are comparable, however, tributyrinase is much more prone to deactivation in the presence of an emulsion. The heat of denaturation was reduced to 19.5 kcal/mole, at the same pH level. The lipase fraction was not included in this investigation.

In a study carried out by Gili et al., ground wheat germ was treated with hot air in a fluidized bed dryer [35]. The heat-treated particles were subsequently assayed for lipase activity *via* wetting and thermostating at 40 °C for 48 h, followed by the extraction of the germ oil and determination of FFA content [36]. It was determined, that heating to 110 °C for 15 min leads to residual activity ($\Delta FFA/\Delta FFA_0$) 69,1%, further lowered in elevated temperatures, and a calculated activation energy of wheat germ inactivation reaction was estimated at 121.74 ± 9.24 kJ mol^{-1}. It is a significantly higher value than the one obtained by Stauffer and Glass for isolated enzymes in an aqueous solution [15]. A possible explanation for this is a protective effect of the original matrix of the enzyme.

A method of the inactivation of enzymes in ground wheat germ *via* continuous microwave radiation was

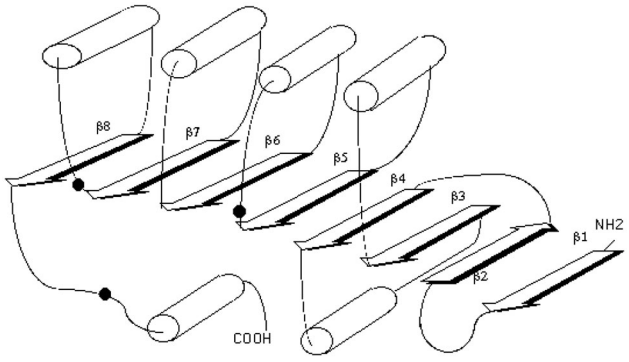


Figure 1. Schematic diagram of α/β WGL fold (Ollis, et al.) [24].

MERRGLLKTA LLACLLVVCS GRVPMVIQQP STTIYNSTLA KTLVEYAAAI YTADLTQLFT
WTCDRCGDLI EGFEMMDIIV DVENCLEAYV GFASDINAVI VVFRGTQENS IQNWIEDLLW
KQLDLDYPGM PEAMVHRGFY SAYHNTTIRD GIVSGIQKTR KLHGDVPIMV TGHSMGAAMA
SFCALDLVVN YGLDDVKLMT FGQPRVGNAA FASYFKRYLP HAIRVTNAND IVPHLPPYFS
FFPQKAYHHF PREVWVHDVG LGSLVYTVEQ ICDDSGEDPA CSRSVSGNSI QDHITYLGVS
MHAEAWSSCR IVMDYAELRY KMDLHGNVVL SKQQQQQPGL SDQRRRHSAQ

Figure 2. Amino acid sequences of WGL.

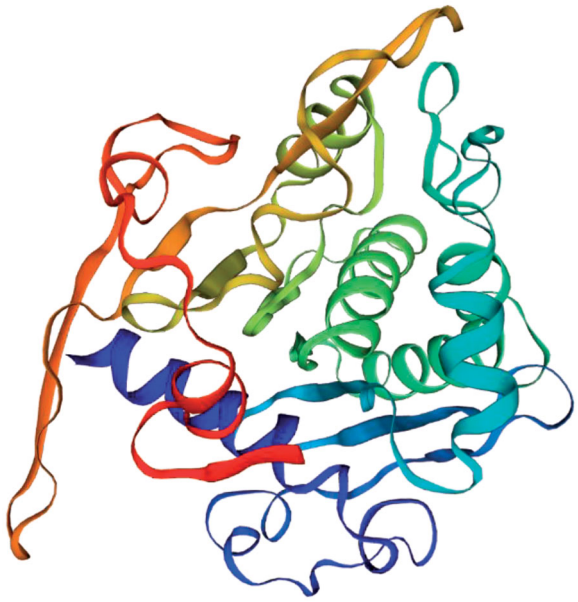


Figure 3. The SWISS-MODEL of WGL.

investigated by Xu et al.[37] The lipase activity before and after the MW treatment was assayed by a modified method of Rose and Pike [34]. It was determined, that within the microwave power range screened in this study, the microwave inactivation of lipase is due to reducing the amount of water, which is required to maintain its catalytic properties, instead of denaturation of the protein. Upon the addition of water to microwaved wheat germ to attain the original water content, the catalytic activity was almost completely restored. This was in agreement with the observations of Kapranchikov, that purified wheat germ lipase retains nearly 30% of its initial activity after heating to 80 °C for 1 h [16].

Rajeshwara and Prakash have investigated the effect of urea on wheat germ lipase in terms of specific enzymatic activity and partial specific volume [38]. In the second part of this study, the stabilizing effects of carbohydrates, glycerol and DMSO were monitored. Wheat germ lipase was purified according to Rao et al.[39] and additionally subjected to Sepharose 6B chromatography. The protein concentration was determined spectrometrically assuming an extinction coefficient $E^{1\%}_{1cm} = 17.5 \pm 0.1$ at 278 nm, according to Rajendran et al. [40], while the partial specific volume was calculated from the density of the solvents and protein solutions according to Casassa and Eisenberg [41]. The enzymatic solution was additionally subjected to ultraviolet and fluorescence spectrometry.

It was observed that urea is a potent inhibitor of wheat germ lipase, and causes reduction of activity by half. The effect is further magnified in the presence of DTT, which causes the cleaving of disulfide bonds in the enzyme structure, and in such case the urea causes a complete loss of activity. Density measurement of the enzyme in the presence of urea shows a decrease in the partial specific volume of the protein, which indicates that inhibitor molecules preferentially interact with it. The quenching of fluorescence and a red shift of the fluorescence emission maxima with increasing urea concentration additionally shows significant conformational changes to sites of tryptophan residues. The effect of urea can be attributed to its preferential interactions with WGL surface and aqueous interface, which leads to exposure of formerly concealed groups to the solution and a progressing unfolding of the structure [42]. A former study performed by the same team indicated that guanidine hydrochloride and guanidinium thiocyanate are even more potent denaturants of WGL, due to their higher preferential interaction parameters [43].

Polyhydroxy compounds and DMSO were demonstrated to increase the thermal stability of wheat germ lipase. Glucose and sorbitol allowed wheat germ lipase to retain up to 41.9% of the original activity, and the thermal stability was directly proportional to their concentration. The effect of sucrose and glycerol was weaker, and DMSO only induced a small stabilizing effect, which was inversely proportional to its concentration. These stabilizing effects have been attributed to preferential hydration of the enzyme molecule, which restricts the movement of the polypeptide chain and allows it to retain an active conformation even at higher temperatures.

Rao et al. have additionally observed the effects of acidic or alkaline pH on wheat germ lipase activity [39,40]. The latter was assayed titrimetrically using triacetin as substrate, and the physicochemical changes to the enzyme were analyzed *via* viscosity measurements, CD, fluorescence spectroscopy and by measurement of sedimentation velocity.

The specific activity of wheat germ lipase was highest at pH 7.6, and was significantly lowered above and below this value, down to near complete deactivation at strongly basic or acidic pH. The viscosity of the protein solution increases in alkaline conditions, which was interpreted as a result of the protein abandoning its elongated shape assumed in neutral pH. The same effect is observed in strongly denaturing conditions, such as in the presence of guanidine hydrochloride. Above pH 8.5, the partial specific volume of the enzyme begins to decrease, the same effect is observed in the presence of urea. This was interpreted to be a result of preferential interaction of the protein with the denaturant and unraveling of the structure of the former. In alkaline pH, fluorescence is quenched as compared to neutral conditions, and a bathochromic shift of the emission maximum is observed. This suggests that the tryptophanyl groups of the molecule are being exposed to bulk solvent, which happens as the structure of the wheat germ lipase deteriorates. Finally, the results of UV circular dichroic spectroscopy confirms that in alkaline pH the protein loses its initial shape, and additionally indicates that the α-helical structures are deformed.

Pancholy and Lund investigated the effect of metal cations on wheat germ lipase, employing 4-methyl umbelliferone butyrate in a fluorometric activity assay [44]. It was observed, that calcium and magnesium salts increase the rate of the reaction, particularly when potassium or sodium salts are also present. Aflatoxin was identified as a potent inhibitor. Copper(II) and ferric acetates caused significant inhibition of the enzyme, and a similar result was observed with the use of

sodium cyanide, sulfide and EDTA. The investigation into the structure of the titular enzyme carried out by Korneeva et al. confirmed that EDTA acts as its noncompetitive inhibitor [45]. Concentrations above 1 uM caused significant loss of activity and nearly complete inhibition at 0.02 M concentration. However, the hydrolysis profile remained unchanged, and subsequent addition of calcium chloride resulted in partial recovery of the enzymatic activity. Kapranchikov et al. observed the inhibitory effect of 10% tannin solution on wheat germ lipase [16].

As is the case with other enzymes, conditions which resemble the native environment of wheat germ lipase promote high activity, whereas extreme temperatures, pH, chelating reagents or metal salts lead to loss of its function. It is imperative to elucidate the inhibitors and activators of this enzyme for its rational application in biotechnology and industry.

Kinetics of wheat germ lipase catalysis

In discussing wheat germ lipase, the latter term is used to describe a variety of hydrolytic activities demonstrated by all the enzymes carried through the commercial purification process. Which one of them is engaged in a given study depends not only on the substrate used in the activity assay, but also its physical state. The kinetic studies carried out by Stauffer and Glass on the separated enzyme fractions led to the conclusion that each has distinct optimal pH values, and responds differently to the presence of an oil-water interface [15].

The tributyrinase fraction was most active at pH $6.6-6.8$ and lipase at 8.0, and the relative reaction rates of both rapidly declined above and below the respective optima. The esterase fraction had a much broader relative rate peak, possibly highest at 7.2, and lost only some of the optimal activity above pH 7.5 and below pH 7. This was explained by the persistent heterogeneity of the fraction.

The tributyrinase and esterase significantly differ in their activity in the absence and presence of an oil-water interface. Aqueous ethyl butyrate was not appreciably hydrolyzed by the tributyrinase unless enough substrate was added to form a separate phase. On the other hand, ethyl propionate was measurably hydrolyzed by the esterase fraction even in unsaturated aqueous solutions.

At last, it was demonstrated that the substrate preferences of the obtained enzyme fractions are also different. The esterase hydrolyzes acetate esters much more efficiently than tripropionin. Tributyrinase is most active toward 4–5 carbon fatty acid esters, does not measurably hydrolyze ethyl acetate, and its rate of reactions with ethyl propionate and triacetin are similar. The lipase was most effective on triolein and did not appreciably hydrolyze acetate esters.

The presence of both esterolytic and lipolytic enzymes in the commercially prepared WGL can be beneficial in applications which require broad substrate specificity, like the hydrolysis or transesterification of oils and oil waste. In studies of wheat germ lipase promiscuity, it is possible that only one of the constituent enzymes engages in the reaction, however results published thus far do not discuss the effects of their preliminary separation.

Immobilization of wheat germ lipase

Enzymes have advantages over homogeneous chemical catalysts due to their biocompatibility, biodegradability, and environmental acceptability in the synthesis of organic products. Lipases are one of the most extensively used enzymes, and play an important role in biotechnological and industrial processes, including the pharmaceutical and food industries [46]. However, their aqueous solubility and instability complicate their applications in organic synthesis. These drawbacks of free enzymes can be overcome by immobilization of enzymes, which is known to improve the properties such as activity, stability, and reusability [47,48].

So far, enzymes have been immobilized on different carriers, including: hydrogels, biopolymers, and inorganic supports. For the immobilization of: lipase, hydrophobic sol–gel [49], chitosan [50], chitin [51], agarose [52], mesoporous silica[53] and metal organic frameworks (MOF)[54] have been employed.

Kosugi et al. reported the esterification of glycerol with high FFA rice bran oil carried out with wheat germ lipase immobilized on an anion exchange carrier. The yield of monoglyceride was good when both esterification and transesterification activities were made to work in parallel. The decrease in the free fatty acid content during reaction with immobilized wheat germ lipase conducted at 40 °C reached 40.1% [55].

Liquid membrane emulsions made of paraffin were used to encapsulate wheat germ lipase [56]. This technique allowed the combination of enzymatic hydrolysis of *rac*-4-acetoxy-cyclopentenone with a selective transport through the organic phase constituting the membrane. Stable emulsions could be prepared with a paraffin membrane containing: cyclohexane (10%), Span 80 (9%), and methyl trioctyl ammonium chloride (TOMAC, 1%). This anion exchanger is necessary to transport the acetate ions, formed during the enzymatic

reaction, through the membrane. Prepared enzyme emulsions were stable when no phase separation is observed within 24 h.

Kloosterman et al. investigated the lipase from wheat germ covalently immobilized on agarose which revealed to be less active in the hydrolysis of octa-*O*-acetyl-sucrose than the native protein [57].

Lipase from wheat germ was covalently bound to the graft copolymer prepared by coupling of poly(acrylic acid-co-acrylamide) with poly(*N*-isopropylacrylamide) using a water-soluble carbodiimide [58]. Immobilized enzyme exhibited so-called "*turn-over*" phenomenon in its catalysis, thus providing sensing device which is responsive to external stimuli.

Reetz et al. reported a very efficient method based on lipase immobilization in hydrophobic silica matrix by sol-gel processing [59]. It was shown, that entrapment of lipases in silicon-containing matrices obtained by hydrolysis of alkoxy-silicon compounds in the presence of appropriate catalysts (e.g. sodium fluoride or potassium fluoride) as well as other additives (e.g. poly(vinyl alcohol, Celite®), resulted in biocatalysts with extraordinarily high catalytic activities. In lauric acid esterification with 1-octanol in 2,2,4-trimethylpentane, the activity was increased by more than two orders of magnitude compared with native lipase. Moreover, the immobilized lipases remained active upon storage for weeks, and were reused without significant loss of activity up to several reaction cycles performed in organic solvents.

Similarly prepared, immobilized enzyme from wheat germ has been successfully used in 3,5-diketoester transesterification [60], as well as in an enantioselective hydrolysis of aryl-(3,4,5-trimethoxybenzylcarbamoyl) methyl ester [61].

Sharma and Gupta observed a 4–5-fold activity increase in *p*-nitrophenyl palmitate hydrolysis upon the encapsulation of wheat germ lipase in alginate [62]. A similar pivotal impact of the alginate encapsulation in both hydrolysis and esterification of various compounds was observed by the teams of Koszelewski and Pierozan [61, 63]. It is also worth noticing, that the immobilized enzyme remained stable after 21 days of storage at 4 °C.

The self-co-immobilization of the wheat germ lipase in the presence of cross-linking glutaraldehyde resulted in a spherezyme, which maintained 40% of native activity toward *p*-nitrophenyl butyrate as the substrate [64].

Lipase from wheat germ was also attached on a porous polypropylene particle carrier (Accurel MP100) with the aid of the cross-linking agent glutaraldehyde [65]. The yield of immobilization reached a high level of 98%. Effectiveness of immobilized lipase was improved using reduced size Accurel particles. Pretreatment of carrier by immersion in ethanol was an important process to modify the porous surface of the polymer. The amount of lipase adsorbed on the Accurel surface pretreated with ethanol was twice as high as on an untreated carrier. Furthermore, an obtained catalyst was used in a packed reactor for triolein hydrolysis under flow conditions with supercritical carbon dioxide as the solvent [66].

Jędrzejewska et al. have studied the enzymatic hydrolysis of ethyl 3-hydroxy-3-phenylpropanoate. They have shown, that direct adsorption of the wheat germ lipase on gold nanoparticles (AuNPs) was an inefficient immobilization method, while covalent attachment to the gold nanoparticles capped with α-lipoic acid resulted in an active catalyst [67].

Macroporous epoxy-activated silica gel revealed to be a cheap, safe and very efficient carrier for immobilization of wheat germ lipase [68].

Recently, nylon-6-grafted with PGMA was used as a carrier for enzyme immobilization [69]. The immobilized enzyme showed higher stability compared to the native enzyme. The immobilized enzyme retained 18% of its activity in *p*-nitrophenyl palmitate hydrolysis after being recycled 8 times. In a storage stability test, immobilized lipase was able to retain 70% of its activity after being stored for 30 days, while free enzyme activity decreased to 15% after 20 days of storage.

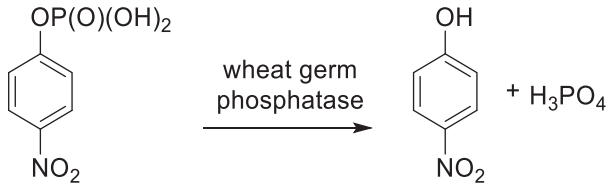
The strategy for immobilization has important consequences for activity and stability of the enzyme. Among the factors that need to be considered are the position for attachment, the carrier type and cross-linking chemistry. Therefore, as a practical matter, it is necessary to carefully screen different immobilization conditions to optimize the activity and stability of any given enzyme.

Other examples of wheat germ hydrolases

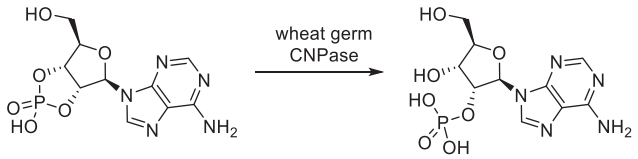
Crude wheat germ lipase prepared by the Singer method is far from specific toward reactions of forming or breaking the ester bond. The evidence of the presence of other hydrolases was first provided by Brouillard et al. as well as by Fink et al. The two teams determined that chromatography and electrophoresis of commercial wheat germ lipase produces several zones of other enzymatic activities - toward phosphates and esterified mono- and disaccharides [70,71]. Several other wheat hydrolases have been eventually separated and investigated.

Kawarasaki et al. have described a method for the separation of six isoenzymes of wheat germ

Entry	Carrier	Immobilization	Substrate	Reaction	Activity	Ref
1	Dowex MWA-1	absorption	glycerol, fatty acid	esterification	Retained	[55]
2	Liquid membrane	encapsulation	*rac*-4-acetoxy-cyclopentenone (HCP)	hydrolysis	–	[56]
3	Agarose	covalent attachment	octa-*O*-acetyl-sucrose	hydrolysis	Decreased	[57]
4	Graft copolymer	covalent attachment	triacetyl glyceride	hydrolysis	Retained	[58]
5	Sol-gel	encapsulation	a) lauric acid/1-octanol b) 3,5-diketoesters c) aryl-(3,4,5-trimethoxybenzylcarbamoyl) methyl ester	esterification/ hydrolysis	Increased	[59–61]
6	Sodium alginate	encapsulation	a) *p*-nitrophenyl palmitate b) aryl-(3,4,5-trimethoxybenzylcarbamoyl) methyl ester c) butyric, lauric and oleic acids/ tributyrin, coconut and olive oils	esterification/ hydrolysis	Retained	[61–63]
7	Self-immobilization	cross-linking	*p*-nitrophenyl butyrate	hydrolysis	Retained	[64]
8	Accurel MP100	absorption	a) triolein	hydrolysis	Retained	[65,66]
9	Macroporous silica gel	covalent attachment	triacetin	hydrolysis	Retained	[68]
10	Gold nanoparticles	covalent attachment	ethyl 3-hydroxy-3-phenylpropanoate	hydrolysis	Retained	[67]
11	Nylon-6-	covalent attachment	*p*-nitrophenyl palmitate	hydrolysis	Retained	[69]



Scheme 2. Wheat germ phosphatase catalyzes hydrolysis of 4-nitrophenyl phosphate.



Scheme 3. 2′,3′-Cyclic mononucleotide 3′ phosphodiesterase (cyclic nucleotide phosphodiesterase, or CNPase) isolated from wheat germ.

phosphatase from an initially isolated mixture of enzymes described by Singer (Scheme 2) [72]. They operated efficiently in acidic solutions (pH 5.5 to 6.0) and hydrolyzed nucleotide di- and triphosphates, as well as phosphoenolpyruvate and 4-nitrophenyl phosphate. However they were inactive toward nucleotide monophosphates, glucose 1-phosphate, and glucose or fructose 6-phosphate. Interestingly, none of the obtained isozymes lost their activity upon the addition of chlorhexidine, an ATPase inhibitor [73]. Only three of them were able to hydrolyze phosphotyrosine.

To the extent of our knowledge, wheat germ acid phosphatase has not been applied in (chemo)enzymatic synthesis of pharmaceutically relevant molecules, although the substrate specificity of its isoenzymes and its reaction mechanism toward representative phosphate esters and the influence of immobilization have been extensively covered [74,75].

Tyc et al. have described the purification and characterization of a 2′,3′-cyclic mononucleotide 3′-phosphodiesterase (cyclic nucleotide phosphodiesterase, or CNPase) from wheat germ (Scheme 3). It selectively hydrolyzes 2′,3′-cyclic mononucleotides to nucleoside 2′-phosphates [76]. This enzyme was obtained by the inhibition of proteases in the crude enzyme extract, saturation with $(NH_4)_2SO_4$ and chromatography until SDS-PAGE gave a single peak. The purified CNPase was active toward 2′,3′-cyclic adenosine phosphate and inert toward 2′-, 3′-, and 5′-nucleoside monophosphates and triphosphates. DTT, N-ethylmaleimide and EDTA did not inhibit the reaction.

Wheat germ is also a source of: phytase [77], ribonuclease H [78], arylesterase [79], protein-serine/threonine phosphatase [80], and many other hydrolases, however their description is outside the scope of this review. It is clear, that thanks to the economic advantages of wheat germ, these too will soon draw the interest of biotechnology.

Current use of WGL in biotechnology, biosynthesis and organic synthesis

The biotechnological application of WGL

Wheat germ is rich in: essential amino acids, minerals and vitamins, and has become an attractive ingredient in the production of food and diet supplements. For the latter purposes, enzymes present in the germ need to be inactivated in order to prevent rancidity and extend the shelf life. We would like to direct the reader to a comprehensive review on application of wheat germ in the food industry by Boukid et al.[10]

Wheat germ lipase has been described as a promising biocatalyst for the preparation of biodiesel from waste oils and oils unsuitable for food production. The enzymatic approach to fuel production has significant advantages – lipases are highly selective, and under optimized reaction conditions can efficiently produce fatty acid esters of monohydroxy alcohols from both glycerides, even at a significant concentration of FFA. Additionally, lipases react under mild conditions, and several of them can be effectively immobilized and reused. Hydrolysis with alkaline metal hydroxides and alkoxides, is plagued by side reactions – mainly saponification, which causes an emulsion to form, and leads to difficulties in product recovery and production of excess alkaline wastewater. Heterogenous acidic or basic catalysts requires high temperatures and pressures, and are sensitive to FFA. The observations made by Pierozan et al. [63, 81] elucidate some of the catalytic properties of wheat germ lipase in esterification reactions. A general preference of the enzyme was observed toward short and medium chain fatty acids and alcohols with two or three carbon chain lengths, precursors of esters suitable for biodiesel formulation.

A patent submitted by Robert Menzi describes a process for production of pasta utilizing wheat germ lipase as one of the proposed enzymatic additives [82]. An important organoleptic property of cooked pasta is its elasticity, absence of sticking, and lack of a starch residue on the surface. In raw pasta, granules of starch are dispersed in the protein network. When boiled in hot water, they absorb water and increase in size, while the surrounding protein denatures and is prone to lose its mechanical properties. Eggs, which are a common additive to pasta dough, strengthen the protein interface, prevent sticking and smooth the surface of cooked pasta. As claimed in the patent, the addition of wheat germ lipase to a flour mixture instead of eggs, improves the elasticity of such prepared pasta and prevents sticking. The required enzyme quantity is very low, 0.2 to 10 g per 100 kg of flour. Such a formulation would result in a high quality, vegan product, free from chicken egg allergens or egg-borne bacteria.

In a patent submitted by Crinos Industria Farmacobiologica S.p.A. [83], wheat germ lipase was described as an additive to skin and hair care products. Surfactant research is an ever ongoing study with the aim of balancing the properties of cosmetic formulations such as: surface activity, required for efficient washing; avoiding excessive delipidation, drying and irritation of the skin; desirable organoleptic properties; environmental impact; cost and ease of manufacturing. The addition of small quantities ($0.1-1\%$ w/v) of Pharmacopeia grade wheat germ lipase was described to improve cleaning properties, and reduce the required amount of synthetic detergents to be added to the formulation, without a significant loss of detergency. Binding the enzyme to a carrier such as agarose was proposed to resolve the issue of limited stability of WGL. The suggested formulation of a skin cleansing lotion, containing 1% of plant lipase, was proposed to be pH-adjusted by the addition of EDTA. The latter is however an inhibitor of wheat germ lipase, which raises the question whether the cleaning properties of wheat germ lipase in a cosmetic formulation is intended to be the result of its catalytic activity, or the surface activity of the inactivated enzyme.

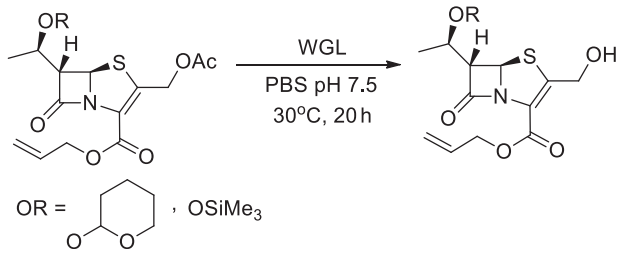
Khor et al. have described a method for preparation of FFA from palm oil using commercially available wheat germ lipase [84]. The enzyme was assayed by solubilizing it in Tris-HCl buffer and incubating it with palm oil dissolved in hexane. This was followed by extraction of products with acetone and ethanol. The amount of liberated free fatty acids was estimated by direct NaOH titration and the activity of wheat germ lipase was estimated at 20 nmol FFA $mg^{-1}\,min^{-1}$.

The application of WGL in hydrolysis reactions

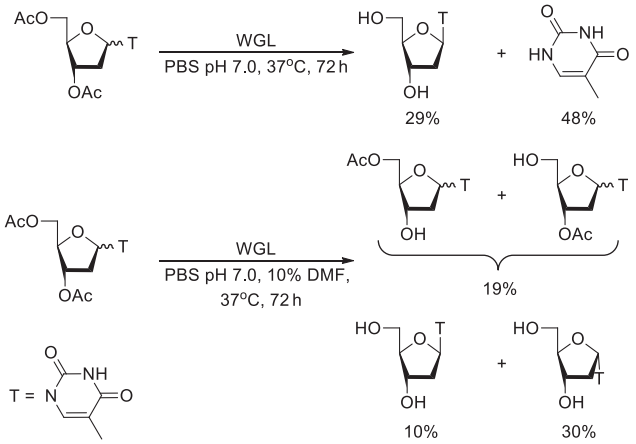
The crystallographic description – not to mention the tertiary structure – of wheat germ lipase remains unavailable, and the best approximation is the aforementioned simulated structure provided by SWISS-MODEL. Therefore, the application of this enzyme in chemical research is largely an empirical study. Experiments are planned on the basis of its already discovered activities, rather than being designed from the knowledge of the dimensions, charge distribution and conformational flexibility of its catalytic center. It has encouraged the search for many new activities and applications in organic chemistry, as described below.

Altamura et al. have successfully employed wheat germ lipase (among other lipases) in regioselective hydrolysis of a diester intermediate in the synthesis of two penem antibiotics, and achieved the desired monoester with an 80% yield (Scheme 4) [85]. The enzymatic approach omitted the use of expensive trialkylsilyl chlorides for protection.

The application of wheat germ lipase as a biocatalyst allowed for a degree of selectivity in the hydrolysis of a challenging group of substrates, peracetylated furanose and pyranose nucleosides [86]. From a mixture of alfa and beta anomers in a 2:1 respective ratio, only a completely deacetylated beta anomer was obtained in 29% yield when the reaction was carried out in PBS at pH 7



Scheme 4. Regioselective hydrolysis catalyzed by WGL.

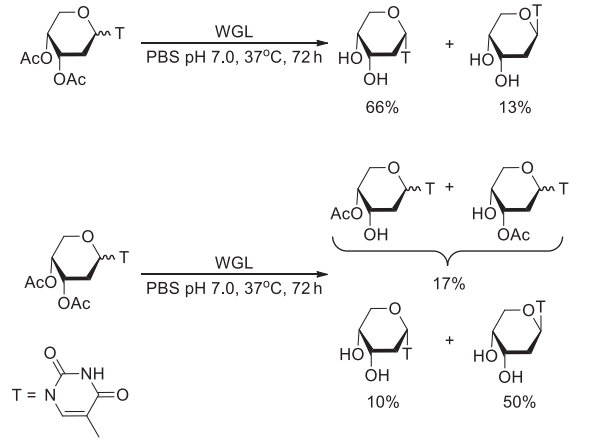


Scheme 5. Chemoselective deacylation of furanose catalyzed by WGL.

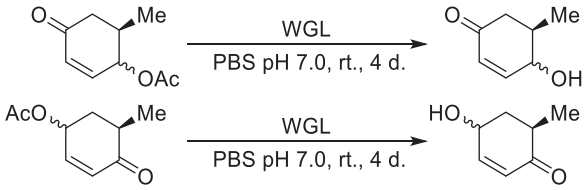
(Scheme 5). The only side product observed under these conditions was free thymine. A change in stereoselectivity was observed upon the addition of 10% DMF. In this case the hydrolysis reaction resulted in a mixture of mono- and deacetylated furanose nucleosides. Fully deacetylated anomers were obtained in an alpha:beta 3:1 ratio, however the formation of free thymine was not observed.

The outcome was similar in the case of pyranose nucleosides, however the observed yields were 79% in pure PBS and the obtained deacetylated product was a mixture of anomers in the ratio alpha:beta 5:1 (Scheme 6). Again, the addition of the same proportion of DMF resulted in the product containing a minor amount of monoacetylated pyranose nucleoside. In case of these substrates, the release of free base thymine was not observed.

Meister et al. have used wheat germ lipase in the synthesis of natural product building blocks, monomethylated 4-hydroxycyclohexenones [87]. This biocatalyst was used in place of inorganic bases for monohydrolysis of an intermediate acetate ester (Scheme 7). This was conducted in order to carry out the reaction under mild conditions, due to the decomposition of the product or substrate in the presence of strong bases. The application of WGL in a phosphate buffer (pH = 7) in room temperature allowed to obtain the required



Scheme 6. Chemoselective deacylation of pyranose catalyzed by WGL.

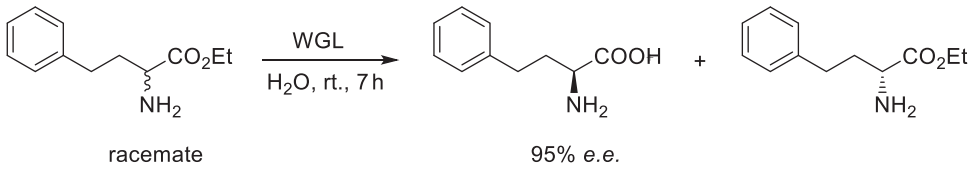


Scheme 7. Enzymatic hydrolysis of 4-hydroxycyclohexenones catalyzed by WGL

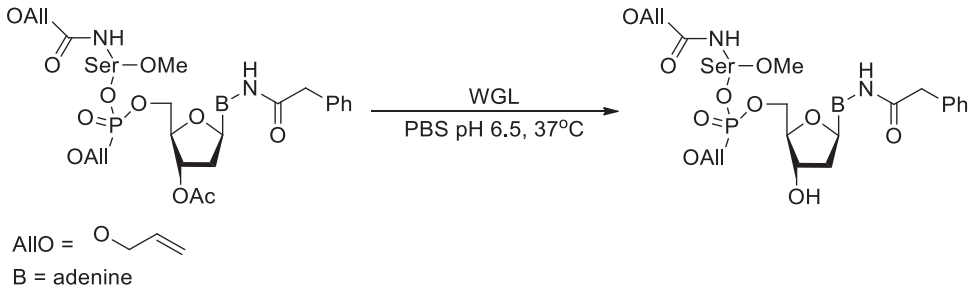
substituted cyclohexenones in 76% – 88% yield, although not as single diastereoisomers.

Wheat germ lipase was one of the lipases applied in a facile enzymatic resolution of homophenylalanine, for the purpose of the synthesis of tripeptide clinical drugs used for hypertension treatment [88]. This pathway has the advantage of avoiding the use of protective groups or prolonged reaction times, which were features of previously described synthetic procedures of this amino acid. The investigated biocatalysts led only to the reaction of the L-enantiomer of homophenylalanine ethyl ester, leaving intact the D-enantiomer of the ester, which can be extracted with ethyl acetate. The reaction of wheat germ lipase was carried out in water at room temperature, and over the course of 7 h resulted in achieving the highest conversion among the investigated enzymes (85%), and 95% enantiomeric excess (Scheme 8).

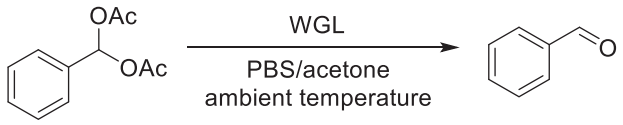
Jungmann and Waldmann have applied a series of enzymes in their investigation into the hydrolysis of protective groups during the synthesis of functionalized nucleopeptides [89]. The main objective in devising a group protection/deprotection stage is to achieve the highest possible selectivity toward the targeted substituents (Scheme 9). A common requirement in case of deprotection reactions is mild conditions and a relatively low reaction temperature, in order to avoid side reactions, not limited to removing other protective



Scheme 8. Enzymatic kinetic resolution of racemic homophenylalanine catalyzed by WGL



Scheme 9. Enzymatic deprotection of functionalized nucleopeptides catalyzed by WGL



Scheme 10. Enzymatic deprotection of acylals catalyzed by WGL

groups. In case of the aforementioned nucleopeptides, wheat germ lipase was discovered to selectively hydrolyze the acyl group present in the carbohydrate ring, in the presence of an allyloxycarbonyl group and an ester bond between phosphorus and the serine group, as well as peptide bonds. The reaction was carried out in a phosphate buffer at pH 6.5, and a yield of 64% was achieved in 37 °C.

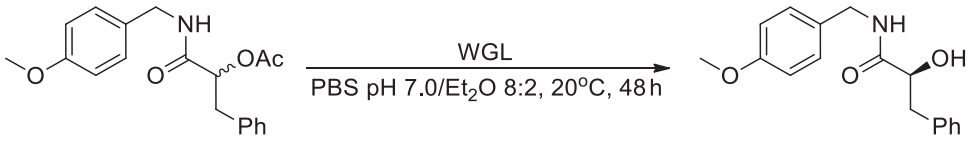
Wheat germ lipase has been also screened in a series of hydrolysis reactions of carboxylic acid esters by Koszelewski et al. It was evaluated in the kinetic resolution of (*E*)-methyl 5-(phenyl)-5-acryloyloxypent-2-enoate, and the reaction yield was 37% – the same as when lipase from *Pseudomonas fluorescens* was used [90].

1,1-Diacetals (acylals) are an emerging group of molecules, that serves as a form of protected aldehydes and are more stable than acetals. Wheat germ lipase was one of the hydrolases investigated during a study of their enzymatic deprotection, which would be a suitable alternative to hydrolysis with strong acids (Scheme 10). The use of this enzyme led to 53% conversion of benzylidene 1,1-diacetate to the parent benzaldehyde [91].

Wheat germ lipase was demonstrated to be an efficient catalyst in the kinetic resolution of α-acetoxy amides obtained in a Passerini reaction by Szymanski et al.[92] The enzymatic hydrolysis resulted in a formation of *S*-enantiomer of the alfa-hydroxyamide with 93% *ee*. The choice of diethyl ether as a co-solvent resulted in significant improvement not only in substrate solubility, but also improved the enantioselectivity of the enzymatic reaction and shortened the reaction time to 48 h. The resulting compound could be efficiently converted into the *R*-enantiomer or its parent *R*-α-acetoxyamide in less than three steps and in a very high yield (Scheme 11). The obtained hydroxyamide was subjected to five highly efficient steps to convert it into a corresponding amino acid with 98% *ee*. Owing to the wide scope of the three component reaction, both natural and synthetic amino acids can be obtained using the described protocol, and very efficient control of the stereogenic center can be achieved. This served as a starting point toward a chemoenzymatic synthesis of tripeptide mimetics and *N*-methylated protected peptides [93,94].

The influence of the co-solvent was further investigated in a following study of the enzymatic resolution of α-acetoxy-α-benzylamides. During the studies, a clear correlation between hydrophobicity of the solvent (expressed in terms of logarithm of the partition coefficient) and the reaction enantioselectivity was observed. Diethyl ether remained the optimal choice of a co-solvent for this reaction setup. Replacing wheat germ lipase with Amano PS resulted in moderate improvement in yield, however at the expense of enantioselectivity, and a much longer reaction time [95].

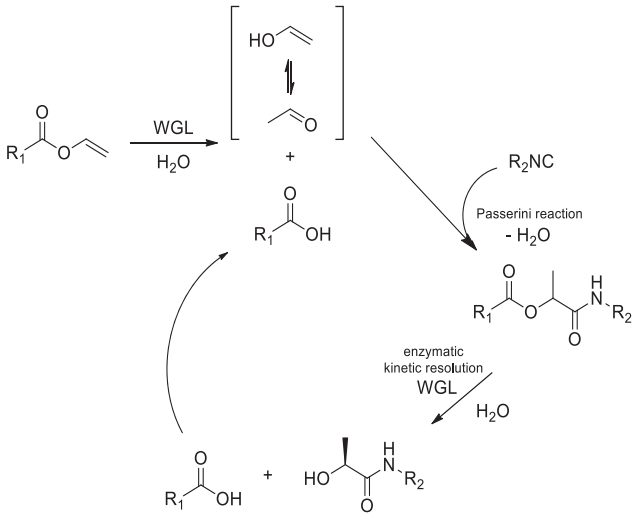
On the basis of the above investigations, Xia et al. have studied the activity of wheat germ lipase in organic solvents, and the influence of an acyl group donor, temperature and water activity [96]. It was



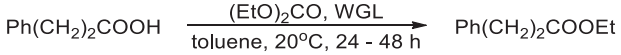
Scheme 11. Enzymatic kinetic resolution of racemic α-acetoxy amides catalyzed by WGL

determined, that in *n*-hexane wheat germ lipase acts as an anti-Kazlauskas enzyme, and requires a high initial water activity in order to efficiently catalyze the resolution of secondary alcohol acyl esters. This was explained as a reflection of changes in the structure of the substrate binding site, and the reduced flexibility of the enzyme when little water is available in its environment. In the case of 1-phenylethyl acetate enantioselectivity was greater than 200, and the obtained conversion ratio was 27%, which reduced to 0 when the initial water activity was less than 0.55. The effect of temperature on enantioselectivity became apparent only above 50 °C and there was no significant effect of the acyl group donor chain length.

Żądło-Dobrowolska et al. have tested wheat germ lipase in a multicomponent synthesis of enantiomerically pure α-hydroxycarboxamides in a tandem multicomponent reaction [97]. In this case, investigated lipases played a part in both stages of the process. One of them is the *in situ* hydrolysis of vinyl alcohol esters into substrates of the Passerini reaction, which was planned to avoid using a low boiling and irritant acetaldehyde. The other was the kinetic resolution of the α-acyloxy carboxamides obtained as a result of the multicomponent reaction. The carboxylic acid is consumed in the Passerini reaction and subsequently regenerated in the enzymatic hydrolysis of its products, which led to increased conversion, however at the expense of enantioselectivity. The latter was demonstrated by confronting results of the whole tandem reaction and those of kinetic resolution of the racemic Passerini product (Scheme 12). The use of wheat germ lipase in pH 7.4 phosphate buffer resulted in 73% yield of the target alpha-hydroxy carboxamide, and the highest enantiomeric excess of 26% among all investigated lipases (in favor of the S-enantiomer).



Scheme 12. Enantioselective tandem multicomponent Passerini reaction catalayzed by WGL

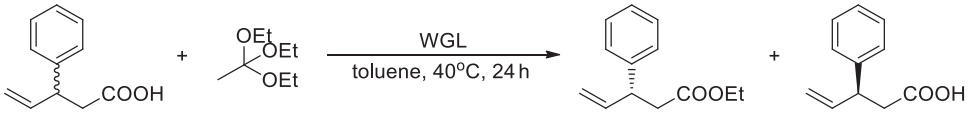


Scheme 13. Carboxylic acids esterification using various alkoxy group donors catalyzed by WGL

The application of WGL in esterification reactions

Wheat germ lipase was also successfully applied as a catalyst in the esterification reaction of carboxylic acids using various alkoxy group donors (Scheme 13). Dialkyl carbonates are alkoxy group donors seeing ever wider use in esterification reactions, thanks to the shift in reaction equilibrium from the resulting carbon dioxide release. Their application was extended as well to enzymatic esterification in non-aqueous media with the use of a combination of enzymes – wheat germ lipase, acylase I from *Aspergillus melleus*, crude papain powder, Amano AK lipase from *Pseudomonas fluorescens* and Novozym 435. Since the presence of trace amounts of alcohol was demonstrated to not be able to start the reaction course, the free hydroxy groups present in the structure of enzymes served as an explanation for the reaction mechanism. However the specific role of wheat germ lipase was not clarified in this case [98].

Other alkoxy group donors accepted for wheat germ lipase are trialkyl orthoesters [99,100] and acetals [101]. These compounds were successfully applied for enzymatic kinetic resolution of racemic 3-phenyl-4-pentenoic acid catalyzed by WGL (Scheme 14). The best results were obtained for the reaction of racemic 3-phenyl-4-pentenoic acid with triethyl orthobenzoate in toluene in the presence of WGL. The product was obtained in 50% yield with 90% enantiomeric excess. The developed kinetic resolution was later applied in a stereoselective syntheses of (S)-3-phenylGABA hydrochloride, as well as (S)-4-phenyl-2-pyrrolidone [101].



Scheme 14. Enantioselective esterification of carboxylic acids using various alkoxy group donors catalyzed by WGL

The above results leave no doubt that wheat germ lipase will continue to find application in regio-, enantio-, and diastereoselective hydrolysis, acylation and transesterification reactions.

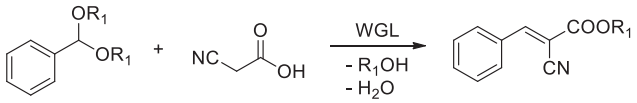
Promiscuous activity of WGL

Enzymes also have the ability to catalyze different reactions than those contributing to their known physiological activity [102]. This property is called enzymatic promiscuity, and can be divided into three types. Substrate promiscuity manifests itself when the enzyme catalyzes its native reaction on non-natural substrates. Condition promiscuity refers to the enzymatic catalysis of the native reaction on natural substrates, but in conditions other than native – among others, different solvents and temperature. As shown in the previous chapter, wheat germ lipase can demonstrate both these types of promiscuity.

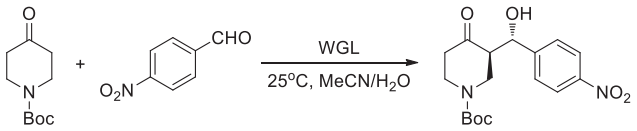
Here, we will show examples of its catalytic promiscuity, which is the ability to catalyze reactions differing in mechanism from the one contributing to the physiological activity of the enzyme [91]. The activity of wheat germ lipase in the reactions described below have been verified by comparing the results with those achieved with a thermally or chemically denatured enzyme, or excluding the biocatalyst altogether. In all cases, the obtained yield or stereoselectivity were suppressed.

Knoevenagel condensation

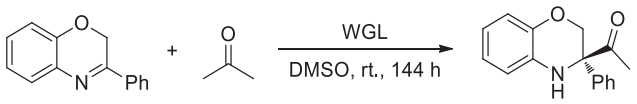
Following the development of an enzymatic Knoevenagel condensation [103], the group of Ostaszewski demonstrated a cascade involving esterification of cyanoacetic acid with benzaldehyde dimethyl acetal, followed by condensation leading to a single isomer of 2-benzylidene cyanoacetic acid methyl ester [104]. Both steps of the reaction were catalyzed by a single enzyme (Scheme 15). Wheat germ lipase was one of the hydrolases investigated in the screening, and its use offered a 21% reaction yield. This clearly demonstrates wheat germ lipase is capable of multi-promiscuous activity under suitable conditions.



Scheme 15. Tandem chemoenzymatic Knoevenagel condensation catalyzed by WGL.



Scheme 16. Aldol reaction catalyzed by WGL.



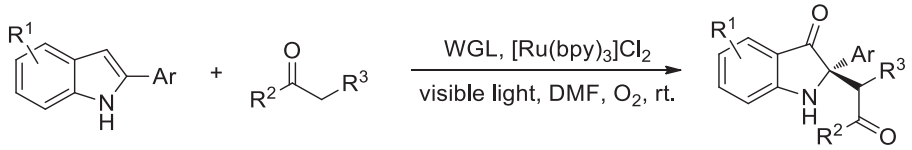
Scheme 17. Mannich reaction catalyzed by WGL.

Aldol condensation and Mannich reaction

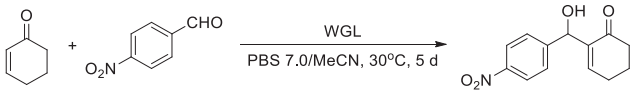
As mentioned in the introduction to this review, Guan et al. investigated the use of lipases in the stereoselective aldol condensation of Boc-protected 4-piperidone [1]. The use of wheat germ lipase resulted in the formation of corresponding product with 28% yield and 12%ee (Scheme 16).

This work was followed up by a paper by Wu et al., in which wheat germ lipase proved superior to PPL II and other investigated lipases in a Mannich reaction of 3-phenyl-2*H*-1,4-benzoxazine with acetone [105]. The reaction was carried out in DMSO at room temperature within 144 h, and the target compound could be obtained in 51% yield with 86% enantiomeric excess (Scheme 17).

The application of WGL as a stereoselective Mannich reaction biocatalyst was further extended in a tandem reaction leading from 2-arylindoles to 2-disubstituted 2-arylindol-3-ones [106]. It consisted of a photooxidation stage catalyzed by a tris(2,2′-bipyridyl)ruthenium(II) photocatalyst in the presence of oxygen and visible light, followed by a biocatalytic reaction between the obtained 2-arylindol-3-one and a ketone (Scheme 18). The structures of the 2-arylindole substrates highly influenced the yield of the reactions, and were significantly lower if the aromatic rings at the 2-position were



Scheme 18. Photo-enzymatic reaction catalyzed by WGL



Scheme 19. Morita-Baylis-Hillman reaction catalyzed by WGL

substituted with electron-withdrawing groups. However in most of the investigated cases, the enantiomeric ratio of the tandem reactions products were above 80:20, in favor of the *S* enantiomers.

Morita-Baylis-Hillman reaction

Reetz et al. [107] have observed activity of wheat germ lipase in a reaction between Michael acceptor cyclohexenone and 4-nitrobenzyl aldehyde (Scheme 19). In the same conditions, the standard B lipase from *Candida antarctica* showed no activity in this reaction. It is assumed that the serine group of the enzyme could act as the nucleophile and reversibly bind to the β-carbon. It does not necessarily need to be the Ser side chain forming the catalytic triad, as the reaction was also successfully carried out with a non-enzyme protein BSA.

Catalytic promiscuity can greatly enhance the application of enzymes in organic synthesis and industrial applications. Hydrolases from other sources have demonstrated the ability to catalyze Ugi, Hantzsch, Canizzaro and Henry reactions, and contribute to chemoenzymatic processes. The described economic advantages and promising promiscuous activity of wheat germ lipase certainly encourages investigation into (chemo)enzymatic reactions utilizing this enzyme.

Conclusion and perspectives

In this review, we have described the discovery of wheat germ lipase: its structure, stability, activators and inhibitors. We have also shown the kinetics of its reaction with esters, and methods of its immobilization. Finally, we present an overview of its applications in biotechnology, green chemistry, and we have given examples of its promiscuous reactions.

Wheat germ lipase is produced from an inexpensive, widely available and renewable source. Wheat is a staple food crop, nontoxic to humans and does not carry the risk of infection. Wheat germs are a leftover product of grain milling, and have been successfully applied in food production, diet supplements, skin care formulas and animal products. However, they are still an underutilized resource available in very high annual supply. This gives wheat germ lipase several advantages over lipases most commonly used in green chemistry, which are produced from potentially harmful and carcinogenic fungi and bacteria.

Wheat germ lipase can be produced from genetically unmodified wheat, and therefore its applications in biotechnology are not impeded by GMO restrictions. It can be used in the production of: foods, biodiesel, oil-rich waste treatment and bioremediation, and it is the subject of intense studies in other fields of biotechnology. It has been immobilized on various carriers resulting in stable biocatalysts, some of which are more active than the native enzyme. Wheat germ lipase have also proved to be an efficient and stereoselective biocatalyst in several kinetic resolutions reactions. A number of promiscuous reactions of wheat germ lipase have been described, such as aldol and Knoevenagel condensation, Mannich reaction and Morita-Baylis-Hillman reaction. It is very likely, that a new type of catalytic activity of wheat germ lipase will be discovered, leading to valuable compounds under environmentally sustainable conditions.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the National Science Centre, Poland, project OPUS No. 2016/B/ST5/03307.

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